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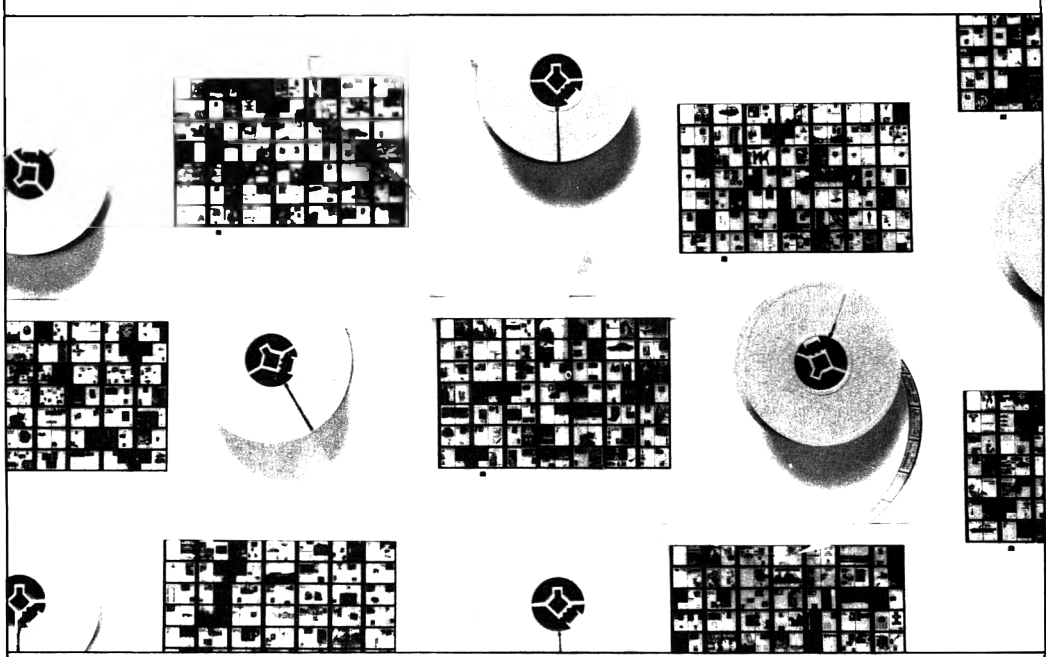
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Plaice Skin Collagen Extraction and Functional Properties

P. MONTERO, C. ALVAREZ, M. A. MARTÍ, and A. J. BORDERÍAS

ABSTRACT

Solutions and mechanical treatments for decolorizing and washing of collagen from plaice (*Pleuronectes platessa*) skin were studied. The best means was high-speed homogenization with 0.4M NaCl. Four ways of stabilizing this collagen were tried, involving freezing and freeze-drying, and partial solubilization with 0.05M acetic acid prior to freezing or freeze drying or without partial solubilization. Only freeze-drying caused reduction in solubility and emulsifying capacity. Viscosity was greatest when samples were pre-solubilized. Emulsifying capacity was not changed when samples were frozen and decreased when they were either freeze-dried or presolubilized. Optimum water-holding capacity occurred in samples which had been previously solubilized.

Key Words: Collagen, plaice skin, functional properties.

INTRODUCTION

FEW REPORTS are published on the utilization of fish collagens other than conversion to gelatin. Soluble collagen from the swim bladder of teleosts (ichthyocolla) was perhaps the first collagen to be characterized (Boedtker and Doty, 1955). Another similar material studied was a soluble form of collagen from shark's swim bladder (isinglass, Leach and Barrett, 1967). Fish collagen acquired some economic importance in the past as glue but has been replaced by synthetic products. Kubota and Kimura (1967) found a special collagen in shark fin, elastoidin, with the special characteristic of cysteine content. Sikorski et al. (1984) reviewed the possibilities of collagen as functional material, and Montero and Borderías (1989a), who reported on collagen for manufacture of casings, as an emulsifying agent, foaming agent, and gelling agent. They also reported that collagen from skin was more soluble than muscle collagen. Studies have examined the effects of concentration, pH and ionic strength to optimize functional properties (Montero and Borderías, 1990, 1991; Montero et al., 1991), and shrinkage temperature of collagen fibers by differential scanning calorimeter (Borderías and Rudski, 1985).

Our objectives were to extract collagen from frozen plaice skins from fish filleting operations, determine its functional properties such as solubility, water-holding ability and emulsifying capacity, and examine various ways of stabilizing the collagen.

MATERIALS & METHODS

THE SKINS WERE OBTAINED from plaice (*Pleuronectes platessa*) using a conventional bladed fish skinner (Cretel Comin Int. S.A., Barcelona, Spain) and then frozen. After the skins were thawed by immersion in water at 4°C ± 1, the water was discarded and skins were minced/washed three times in a refrigerated homogenizer (Innaves, Vigo, Spain) at high speed (6000 rpm) with 0.2 and 0.4M NaCl (collagen:brine/ 1:6, for 10 min at 4 ± 1°C). Each time the brine/waste was removed by filtration. After this step, the remaining dermis was washed with water to remove remaining brine and then drained by wrapping in cheesecloth and squeezing by hand. Resulting samples were mixed with saline solution (ratio 1:6), then different concentrations of H_2O_2 were added (6, 9 and 12% of total volume of mixture) and stirred mechanically for 1, 3, 6 or 12 hr. Color parameters L*, a* and b* were determined with a Hunterlab D25-9 colorimeter.

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The collagen, washed as described, was prepared in four different ways for the stucy of stabilization: (a) by freezing (F); (b) by partial solubilization then freezing (SF); (c) by freeze-drying (L); and (d) by partial solubilization then freeze-drying (SL). Samples were frozen in a tunnel freezer at −40 ± 1°C) or freeze-dried in a Telstar model freeze-dryer (Lioalfa, Terrasa, Spain). Partial solubilization was performed in 0.05M acetic acid at a w/v concentration of 1:1, with continuous stirring for 12 hr at 5°C to produce a partially-solubilized, partially swollen collagen paste. Solubility was determined, after purification, in an acidic medium (0.5M acetic acid) following the method of Montero et al. (1991). All determinations were performed in triplicate.

Apparent viscosity, based upon the method described by Montero et al. (1991), consisted in the measurement of a homogenate of collagen in a Brookfield-LV viscosimeter (Brookfield Engineering NABS, Stoughton, MA) at 12 rpm and a temperature of 9 ± 1°C. All determinations were performed in quadruplicate. Sample preparation for the viscosity testing involved homogenizing the collagen for 1 min with 0.5M acetic acid at a w/v ratio of 1:2, in an Omnimixer (Ivan Sorvall Inc., Norwalk, CT) at setting 7 and stirring continuously for 24 hr at 5°C.

Emulsifying capapcity was determined on samples homogenized 1 min in 0.5M acetic acid (w/v ratio 1:2) in an Omnimixer at setting 9. The mixture was then magnetically stirred for 24 hr at 5°C and emulsified according to the method of Montero and Borderías (1991). All determinations were performed in quadruplicate. Water holding capacity (WHC) was determined on sample heated for 30 min in abundant water (w/v ratio 1:30) at 60°C, following the method of Montero et al. (1991). All determinations were performed in quadruplicate. Heat solubilization was based on the method of Montero and Borderías (1990). This consisted in disintegrating the collagen in water (w/v ratio 1:2) by stirring for 1 min, followed by heating in a Julabo F10 water bath (Julabo Labortechnik GmbH, Selloch, Germany) for 1 hr at 10, 20, 30, 40, and 60°C. Samples were centrifuged for 15 min at 4900xg, then the amount of hydroxyproline in the precipitate and supernatant was determined (Leach, 1960). Results were expressed as per cent soluble collagen with respect to total collagen. All determinations were performed in quadruplicate.

Moisture, cruce protein and ash were determined by AOAC (1990) methods 24003, 24024 and 18021, respectively; fat, by the method of Knudsen et al. (1985) which is a modified version of the method of Bligh and Dyer (1959), consisting essentially in the use of dichloromethane instead of chloroform as solvent, and pH by the method of Vyncke (1981). All these determinations were performed in triplicate.

Results were analyzed statistically by 1- and 2-way analysis of variance according to the different variables involved, using the program Statgraphics (STSC Inc., Rockville, MD). The difference of means between pairs was resolved by confidence intervals carried out with a least significant difference (LSD) range test. The level of significance was established at $P \leq 0.05$.

RESULTS & DISCUSSION

SEVERAL TREATMENTS to remove both muscle residue and all epidermis and to purify collagen from the skins were compared. Scraping off the epidermis to leave only the dermis, which is white and composed chiefly of collagen, was impractical for industrial applications. Placing the whole fish in a revolving drum with roughened walls to remove the epidermis by abrasion, followed by removal of dermis with a fish skinner, affected the muscle, damaged the fillet and did not remove the epidermis uniformly. Precisely set fish skinners to remove first the epidermis containing all pigments, and then the dermis, seemed effective in theory but involved added cost and reduced fillet yield.

Washing fish skins with various concentrations of highly diluted acetic acid to swell the colleganous material for better

Table 1—Hunter color parameters and acid solubility of purified flounder skin[a]

	L*	a*	b*	Solubility[b](%)
Original	34.7 ± 1.9a	0.9 ± 0.1a	3.9 ± 0.2a	85.5 ± 2.0a
0.4M NaCl	78.3 ± 0.5b	−0.7 ± 0.2b	−3.1 ± 0.2b	84.3 ± 2.1a
0.2M NaCl	61.9 ± 3.1c	−0.4 ± 0.4b	−6.1 ± 0.6c	83.2 ± 2.1a
0.4M NaCl, 6% H_2O_2	63.3 ± 0.9c	1.4 ± 0.3c	6.6 ± 0.2d	85.3 ± 1.8a
0.4M NaCl, 9% H_2O_2	65.6 ± 1.6c	1.4 ± 0.4c	9.2 ± 0.7e	89.6 ± 0.2b
0.4M NaCl, 12% H_2O_2	61.7 ± 1.5c	1.3 ± 0.6c	9.4 ± 1.4e	90.2 ± 1.8b

[a] Different letters in the same column indicate significant differences ($P \leq 0.05$) between treatments.
[b] Collagen:0.5M acetic acid (1:20).

Table 2—Proximate composition (%) and pH of collagen samples[a]

Samples[b]	Moisture	Protein	Fat	Ash	pH
F	74.0 ± 1.2a	22.9 ± 0.6a	1.1 ± 0.1a	0.5 ± 0.1a	7.3a
SF	83.3 ± 2.1b	17.5 ± 0.8b	0.3 ± 0.0b	0.2 ± 0.03b	5.6b
L	13.2 ± 0.9c	85.1 ± 2.2c	1.0 ± 0.1a	0.7 ± 0.2c	6.6c
SL	14.5 ± 0.7c	83.4 ± 1.8c	1.0 ± 0.1a	0.1 ± 0.01d	5.1b

[a] Different letters in the same column indicated significant differences ($P \geq 0.05$) in the same property.
[b] F = Freezing; SF = Partial solubilization then freezing; L = Freeze-drying; SL = Partial solubilization then freeze-drying.

Table 3—Functional properties of collagen from flounder skins[a]

	Control	F[b]	SF	L	SL
Solubility[c] (%)	85.5 ± 3.0a	88.6 ± 1.5a	80.2 ± 1.5b	65.2 ± 1.5c	40.0 ± 1.5d
Viscosity (cP)	990 ± 10.1a	989.4 ± 10.7a	8330 ± 49.6b	1315 ± 90.8c	38.7 ± 0.3d
Emulsifying capacity[d]	74.1 ± 1.5a	74.2 ± 3.7a	51.7 ± 3.7b	65.2 ± 3.7c	64.8 ± 3.7c
Water-holding capacity (%)	32.8 ± 2.4a	20.8 ± 1.1b	35.6 ± 1.2ac	21.4 ± 1.2b	39.3 ± 1.1c

[a] Different letters in the same row indicate significant differences ($P \leq 0.05$) in the same property.
[b] Code of samples as in Table 2.
[c] Collagen:0.5M acetic acid (1:20).
[d] g oil/g product.

pigment removal was ineffective. However, homogenization at high speed with solutions of NaCl (0.4M) produced good results. Neutral saline solutions scarcely affected the collagen but solubilized myofibrillar proteins from the muscle that adhered to the inner surface of the dermis. These solutions should have neutral or nearly neutral pH and needed to be changed at least twice to remove the pigment. Neutral saline solution swells the collagen fibers and ruptures hydrogen bonds, loosening the fiber mesh. With this treatment collagen could be purified effectively and decolorized.

A notable increase occurred in the brightness (L* value) of samples treated with 0.2 and 0.4M NaCl, the best results occurring with higher concentration (Table 1). More intense bleaching was attempted by adding various amounts of H_2O_2 for 6 hr (Table 1). Johns and Courts (1977) described the use of H_2O_2 not only to bleach gelatin, but also to induce certain changes, such as breaking covalent bonds after treatment with very concentrated H_2O_2. Various concentrations of H_2O_2 were added (6, 9 and 12%), but in no case were L* values higher than with 0.4M NaCl. In samples stirred 6 hr, the Hunter b* values increased considerably, to 9.4 ± 1.4, indicating yellowing of the product. We concluded that H_2O_2 was not suitable for use with this material. Treatments tending to whiten the sample and purify the collagen did not cause decreases in acid solubility (Table 1). We concluded that the 0.4M NaCl treatment did not induce any undesirable aggregation of the collagen. The yield of purified collagen in relation to total skin (dried weight basis) was 48 ± 5%.

Proximate composition and pH

Proximate analysis and pH were compared (Table 2). All pH values for presolubilized sample (SF and SL) were quite low, due to the effect of the 0.05M acetic acid used. The samples had fat values around 1% because the species from which the skins came had a thick adipose panniculus beneath the skin, which contaminated the samples.

Functional properties of purified collagen

On the basis of prior work (Montero et al., 1991), the effect of collagen preparation on solubility, viscosity, emulsifying capacity, water holding ability and heat denaturation were determined. Solubility of collagen prepared by the different treatments of stabilization in 0.5M acetic acid was compared (Table 3). Results were similar to those reported by Montero (1989) in hake and Montero et al. (1991) in trout; however, our results were higher than those found in food mammals. Johns (1977) reported acetic acid solubility of 22.6% for rabbit-skin collagen, 6.9% for lamb and 0.53% for bull. According to Cheftel et al. (1985), solubility is an important condition for development of other functional properties like viscosity or emulsifying capacity. Freeze-drying resulted in loss of protein solubility ($P \leq 0.05$). Such loss of solubility in freeze-dried samples was expected since this method removes water from the system, which would affect protein structure. Partial pre-solubilization of sample had a negative effect on acid solubility of collagen whether frozen or freeze-dried.

Freeze-drying caused scarcely any changes in viscosity ($P \leq 0.05$), and freezing none at all (Table 3). Viscosity was higher in the frozen, partly solubilized sample (SF), and lowest in the sample that was freeze-dried after partial solubilization (SL). In the first case swelling caused by pre-solubilization may have arranged the collagen molecules to be more favorable to interface with surrounding medium, so that viscosity was maximized. Freeze-drying, however, involves not only freezing but also sublimation of most of the water, probably causing aggregation of collagen molecules, previously altered by swelling and partial solubilization, and hence a sharp reduction in viscosity.

If the data for the collagen were compared with published data (Montero, 1989; Montero et al. 1991), viscosity was much greater in other species like trout or hake. The reason for this would seem to lie not only in the different species, but also in the fact that the skins used in our work came from frozen fish and were frozen again after skinning (this was done to reduce industrial practice). As a result of these freezing processes, the

Table 4—Water solubility (%) of purified collagen[a]

Samples[b]	Temperature (°C)				
	10	20	30	40	60
F	4.0 ± 0.5ax	5.1 ± 0.3ax	36.2 ± 2.0ay	84.0 ± 3.2az	93.0 ± 4.5az
SF	10.0 ± 1.0abx	36.0 ± 2.2by	34.0 ± 2.3ay	90.1 ± 5.0az	94.0 ± 2.1az
L	4.2 ± 0.6ax	5.5 ± 0.3ax	6.1 ± 0.2bx	62.0 ± 1.2by	82.1 ± 2.3bz
SL	16.0 ± 1.0bx	22.1 ± 1.0cx	56.0 ± 2.5cy	58.0 ± 4.0byz	64.0 ± 5.0cz

[a] Different letters (a,b,c) in the same column indicate significant differences ($P \leq 0.05$) between treatments. Different letters (x,y,z) in the same row indicate significant differences between temperatures.

[b] Code of samples as in Table 2.

collagen may have undergone slight alteration involving deterioration of viscosity, although as noted, solubility was not significantly altered. If the data were compared under the same conditions with collagen from pig skin (10 cp), the fish skin data yielded higher viscosity values (Montero and Martí, 1992).

The emulsifying capacity of the frozen samples (F) were not different ($P \leq 0.05$) from control values. The other treatments brought about decreases in emulsifying capacity, coinciding with lower solubility (Table 3). The difference ($P \leq 0.05$) between the two frozen samples (F and SF) cannot be explained in terms of solubility, in the water phase. In this respect Cheftel et al. (1985) reported that only a certain degree of solubilization was required for protein molecules to spread between the aqueous and lipid layers and that a certain degree of denaturation in the protein molecule may, depending on protein, enhance emulsifying capacity. However since 60% of collagen is hydrophobic, it may dissolve in the fat and form emulsions in which the continuous phase is fatty (Bailey and Light, 1989). Partial solubilization (samples SF and SL) did not appear to favor emulsifying capacity. As with solubility, the emulsifying capacity and viscosity of solubilized pig skin was lower (20% less) than in our data (Montero and Martí, 1992).

The values of WHC (Table 3) were similar to those observed in collagen from skin of hake (Montero, 1989) and trout (Montero et al., 1991). Swelling and partial solubilization of the collagen (lots SF and SL) had a positive effect on WHC values, since these were different ($P \leq 0.05$) from lots F and L. No differences occurred between freeze-dried and frozen samples with the same pre-treatment (unsolubilized or partially solubilized samples). The higher WHC values found in partially solubilized samples (SF and SL) may be related to swelling of the myofibrillar protein in the extract, indicating that the protein would be predisposed to more physico-chemical binding of water.

This collagen could be used for binding water in blocks of fish mince during frozen storage. This is are supported by Montero and Borderías (1989b), who found that sensory acceptability of hake tails was greater because collagen content was higher when the muscle had undergone aging through prolonged frozen storage.

Heat denaturation commenced before 30°C and was substantial by 40°C (Table 4). At that temperature the collagen molecules were ruptured and converted into elements of lower molecular weight (Balian and Bowes, 1977). The partially solubilized samples (SF and SL) were sensitive to heat, probably because their collagen molecules were unfolded by prior solubilization. At intermediate temperatures, commencing at 40°C, the freeze-dried sampels (L and SL) showed less heat solubilization than did the frozen samples (S and SF). The reason may be that freeze-dried samples are generally less soluble (Montero, 1989) and require higher temperatures ($\geq$ 60°C) to augment collagen solubilization. If the collagen is intended for incorporation in a reconstituted product, it is important that it should denature at the cooking temperature. Considering that cooking temperature for some foods may be as low as 60°C, frozen collagen would be more suitable than freeze-dried collagen.

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Recovered Protein and Reconditioned Water from Surimi Processing Waste

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ABSTRACT

Micro- and ultrafiltration were utilized for recovery of proteins and for water recycling from commercial surimi processing waste water. The proteins recovered by microfiltration showed highly functional properties and composition comparable with proteins in regular surimi. Surimi with a substitution (10%) of recovered proteins did not diminish in gel hardness, elasticity, color, or water retention. Aerobic plate count, chemical oxygen demand, protease activity, and turbidity in the surimi waste waters were reduced by ultrafiltration. Results indicated a potential for recovering proteins to extend yield and recycling water.

Key Words: surimi, waste, protein recovery, water recycling

INTRODUCTION

PRODUCTION OF SURIMI involves extensive washing of minced fish muscle to remove fat and water soluble substances such as sarcoplasmic proteins, pigments, amines, vitamins, and enzymes. Reduction of sarcoplasmic proteins, in turn, concentrates myofibrillar proteins which are the major components responsible for formation of surimi gels. Minced fish solids (≈40–50%) are lost during washing and dewatering (Adu et al., 1983; Pacheco-Aguilar et al., 1989; Yang and Froning, 1992). Wu et al. (1991) reported that myofibrillar proteins of white fleshed fish were considerably soluble in water. Some of the soluble solids, lost in wash water, could be highly functional myofibrillar proteins. Therefore, it may be beneficial to recover, characterize, and utilize such proteins for food use to increase value and lesson cost of waste water treatment.

Several methods of concentrating soluble proteins from surimi wash water have been investigated. Ultrafiltration is the primary commercial method used for dairy whey recovery and has been studied by the National Food Processors Association and others as a means of recovering protein from surimi wash water (Nishioka and Shimizu, 1983; Green et al., 1984; Swafford, 1987; Pedersen et al., 1987, 1989; French and Pedersen, 1990). Nishioka and Shimizu (1983) developed a means of precipitating proteins from surimi waste water by shifting the pH. This method is simple and low cost but results in complete denaturation of the proteins. Use of an ion exchange (IE) resin-binding approach in the dairy whey industry showed promise in producing a very clean product with superior functionality compared to proteins recovered by ultrafiltration (Honer, 1986). A comparison of the three methods (Korhonen and Lanier, 1991) indicated that the composition of proteins recovered by these methods was nearly identical; however, the yield was lowest with the IE method.

Recycling of processing water is gaining in importance to surimi processors due to rising utility costs, limited water resources, and pollution problems associated with disposal. Considerable research has been reported concerning water reuse and conservation for poultry processing (Sheldon and Brown, 1986; Chang et al., 1989; Chang and Sheldon, 1989a,b; Chang and Toledo, 1989). However, little information is available for surimi processors who need to reduce waste loads and recycle processing water.

Our objectives were to recover and characterize the proteins lost with waste water during surimi processing, as well as to recondition waste water by reducing chemical and microbiological loads.

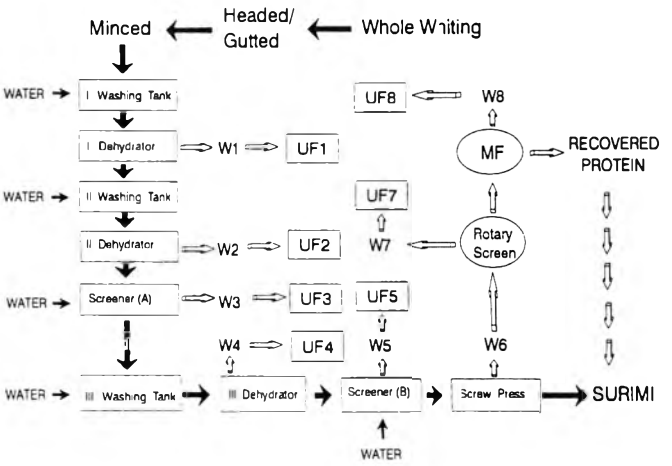


Fig. 1—Surimi processing flow and sample collection. W1 through W8 indicate different waste water streams. UF: ultrafiltration unit. MF: microfiltration.

MATERIALS & METHODS

Sample collection

Sample collection was from in a commercial surimi processing plant during July through September, 1993 (Fig. 1). Whole Pacific whiting (*Merluccius productus*) was headed, gutted, and minced through a screen (5 mm). It was then washed slightly in the first washing tank and more extensively in subsequent tanks (II and III). Screeners were used for both washing and dewatering. Proteins lost in the waste discharged from the screw press (W6) were collected by a rotary screen (100μ) and subjected to microfiltration (MF) with combined screen sizes of 50μ and 30μ (Model MM2, Pure-Grade, Inc., Burbank, CA). The water discharged from the rotary screen and MF unit was labeled as W7 and W8, respectively. All waste water discharged from dehydrators (W1, W2, W4), screeners (W3, W5), rotary screen (W7), and MF unit (W8) were collected for further analysis and for reconditioning of waste water using an ultrafiltration (UF) unit. The UF unit was equipped with a membrane of 30,000 molecular weight cut-off (CUNO Separation Corp., Norwood, MA), selected based on characterization of the molecular weight of whiting protease (Seymour et al., 1994). The quality of recovered protein from the MF unit was evaluated by torsion tests, color measurements, and expressible moisture. The water quality of waste waters and their ultrafiltration permeates were evaluated by measuring the values of aerobic plate count (APC), chemical oxygen demand (COD), clarity (%T), and protease activity. The concentrated proteins collected from ultrafiltration were also characterized.

Surimi gel preparation

To evaluate the functional properties of gels made of MF recovered protein, four treatments were used: (1) 100% surimi (S) control; (2) 100% MF recovered protein (RP); (3) 10% MF recovered protein/90%

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Table 1—Rate and composition of waste waters collected at different po nts on surimi processing line

Waste stream[f]	Waste water/ surimi (L/kg)	Moisture (%)	Protein (%)	Nonprotein n trogen (%)	Fat (%)	Ash (%)
W1	0.46[a1] (0.08[2])	97.10[a] (0.04)	2.34[a] (0.25)	0.13[a] (0.01)	0.19[a] (0.01)	0.41[a] (0.01)
W2	3.75[a] (0.14)	98.78[b] (0.05)	0.97[b] (0.05)	0.08[b] (0.00)	0.11[b] (0.01)	0.38[a] (0.02)
W3	10.07[b] (1.44)	98.87[c] (0.01)	0.99[b] (0.14)	0.04[c] (0.00)	0.06[cd] (0.00)	0.17[b] (0.01)
W4	1.65[a] (0.49)	98.98[d] (0.03)	0.86[b] (0.01)	0.05[c] (0.00)	0.04[c] (0.00)	0.21[c] (0.03)
W5	11.02[b] (6.86)	98.85[bc] (0.03)	0.99[b] (0.01)	0.04[c] (0.00)	0.05[c] (0.00)	0.09[d] (0.01)
W6	2.40[a] (1.42)	99.20[e] (0.03)	0.89[b] (0.01)	0.04[c] (0.00)	0.08[de] (0.01)	0.14[be] (0.00)
W7	2.11[a] (0.88)	99.20[e] (0.04)	0.46[c] (0.06)	0.04[c] (0.00)	0.10[be] (0.01)	0.12[e] (0.01)
W8	0.03[a] (0.01)	99.15[e] (0.04)	0.54[c] (0.02)	0.04[c] (0.00)	0.09[be] (0.01)	0.11[de] (0.01)

[1] and [2] denote mean and standard deviation respectively.
[abcde] Different letters in the same column indicate significant difference ($p < 0.05$)
[f] W1-W8 are sources of waste water as illustrated in Fig. 1.

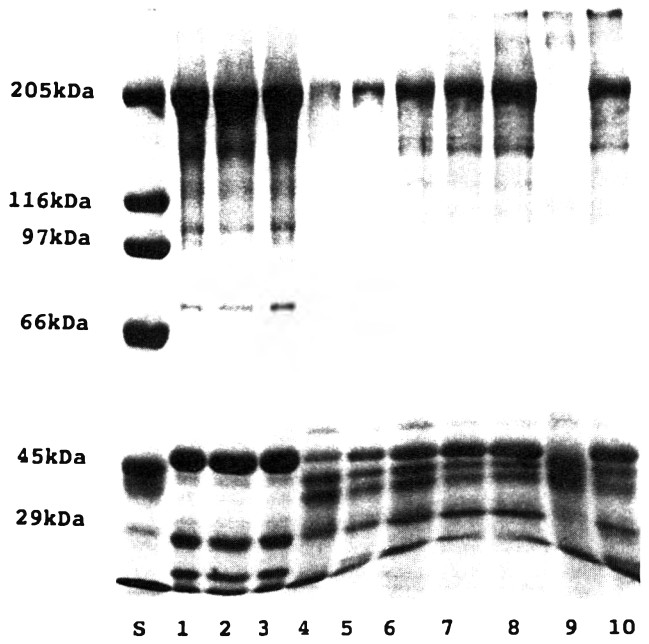


Fig. 2—SDS-PAGE of regular surimi, recovered proteins, and waste water concentrates. S: high molecular weight standard mixture; lane 1 = regular surimi; lane 2 = the proteins collected by rotary screen: lane 3 = the recovered proteins by microfiltration; lane 4–10 = the UF concentrated proteins from waste streams W1, W2, W3, W4, W5, W7, W8, respectively.

regular surimi (10RP/90S); (4) 20% MF recovered protein/80% regular surimi (20RP/80S). All treatments were adjusted to 78% moisture and 2% NaCl. Beef plasma protein (1%, AMP 600N, AMP Inc., Ames, IA) was added as a protease inhibitor. Salt and ice were mixed with surimi and/or MF recovered protein and chopped for 5 min at low speed (1,500 rpm) in a vacuum chopper (Model 5289, Stephan Machinery Corp., Columbus, OH). The temperature during chopping was maintained at 3–5°C using a refrigerated circulator (RTE-100LP, Neslab Instruments, Inc., Newington, NH). The paste was vacuum-packed in a polyethylene bag to eliminate potential air pockets and extruded into stainless steel cooking tubes (i.d. = 19 mm) through a sausage stuffer (Model 14208, The Sausage Maker, Buffalo, NY). Three heating treatments were applied. The paste was heated at 90°C for 15 min in a water bath. In addition, the effect of protease on gel quality was assessed by incubating pastes at 60°C for 30 min prior to heating at 90°C for 15 min. The setting effect was evaluated by setting pastes at room temperature (25°C) for 3 hr prior to cooking at 90°C for 15 min. Gels were immediately chilled in ice water before storing in a refrigerator overnight.

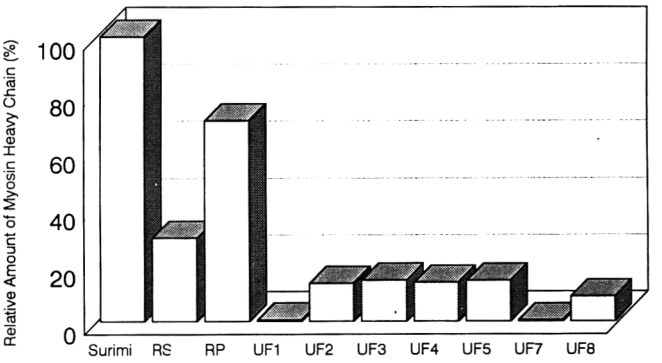


Fig. 3—Relative amount of myosin heavy chain in regular surimi, recovered proteins, and the concentrates of waste waters. RS: protein collected by rotary screen; RP: protein recovered by microfiltration. UF1 through UF8: the UF concentrates from different sources of waste stream as illustrated in Fig. 1.

Torsion test

Torsion tests were performed as described by NFI (1991). Gels were allowed to reach room temperature and were made into hour-glass shapes by a sample milling machine (Gel Consultants, Model 91, Raleigh, NC). Samples were subjected to torsional shear in a gelometer (Gel Consultants, Inc, Raleigh, NC). Shear stress and shear strain, at failure, were calculated using the equations of Hamann (1983). Failure shear stress indicated strength of gels, while failure shear strain denoted deformability of gels.

Color measurement

Color measurements were made by a Chroma Meter (Model CR-310, Minolta Corp., Ramsey, NJ). The instrument, equipped with a D65 illuminant and 2° observer optical position, was standardized for surimi gel measurement by using a Minolta calibration plate (No. 18133009 $Y_{CIE} = 94.5$, $x_{CIE} = 0.3160$, $y_{CIE} = 0.3330$) and a Hunterlab standard plate (Standard No. D44C-1618, $L^* = 82.13$, $a^* = -5.24$, $b^* = -0.55$). Color values were expressed using the International Commission on Illumination L^*, a^*, and b^* values (CIE, 1978). Gels were warmed to room temperature before measurements were made (Park, 1994).

Expressible moisture measurement

The percentage of free water was measured as described by Tsai and Ockerman (1981). Gels (≈0.5 g) were placed on a filter paper (Whatman No. 1) which was set between two pieces of plexiglass and pressed at 35 kg_f/cm^2 by a Carver Laboratory Press (Fred S. Carver Inc., New York) for 1 min. The percentage of free water was calculated by the following equation:

Table 2—Proximate composition of surimi and MF recovered protein

Sample	Moisture (%)	Protein (%)	Fat (%)	Ash (%)
Regular surimi	80.6[1] ± 0.4[2a]	18.3 ± 0.3[a]	0.9 ± 0.1[a]	0.39 ± 0.05[a]
MF recovered protein	84.5 ± 0.7[b]	15.0 ± 0.3[b]	0.8 ± 0.1[a]	0.48 ± 0.04[b]

[1] and [2] denote mean and standard deviation respectively.
[a,b] Different letters in the same column indicate significant difference ($p < 0.05$).

Table 3—Water content of ultrafiltration (UF) concentrates

	UF1	UF2	UF3	UF4	UF5	UF7	UF8
Moisture (%)	91.75[1] ± 0.77[2a]	96.83 ± 0.04[b]	93.30 ± 0.01[c]	96.80 ± 0.14[b]	91.30 ± 0.28[a]	93.35 ± 0.07[c]	93.55 ± 0.49[c]

[1] and [2] denote mean and standard deviation respectively.
[a,b,c] Different letters in the same row indicate significant difference ($p < 0.05$).
[d] UF1-UF8 are ultrafiltration concentrates of different waste waters as illustrated in Fig. 1.

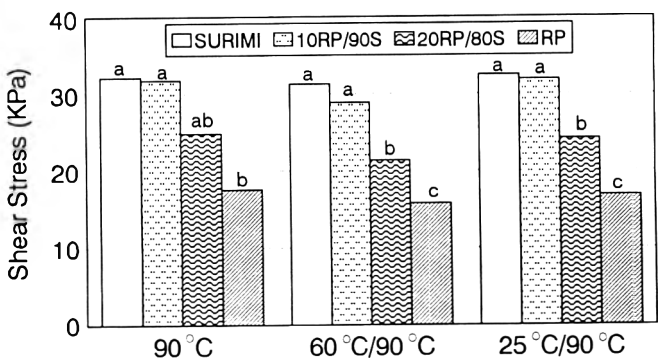


Fig. 4—Shear stress of gels. S: regular surimi; RP: MF recovered protein; 10RP/90S: 10% RP and 90% S; 20RP/80S: 20% RP and 80% S; 90°C: cooked at 90°C for 15 min; 60°C/90°C: 60°C incubation for 30 min followed by cooking at 90°C for 15 min; 25°C/90°C: setting at 25°C for 3 hr before cooking at 90°C for 15 min. Different letters in the same group of bars indicate a significant difference ($p < 0.05$).

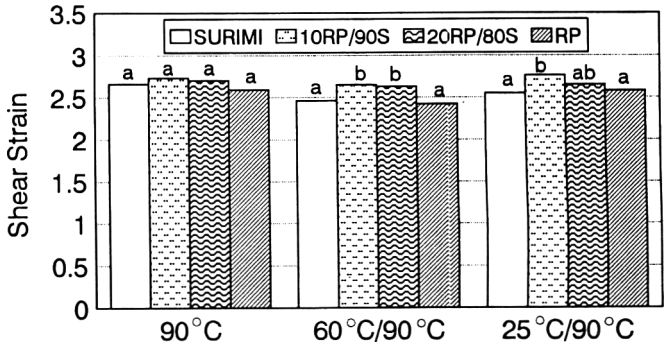


Fig. 5—Shear strain of gels. S: regular surimi; RP: MF recovered protein; 10RP/90S: 10% RP and 90% S; 20RP/80S: 20% RP and 80% S; 90°C: cooked at 90°C for 15 min; 60°C/90°C: 60°C incubation for 30 min followed by cooking at 90°C for 15 min; 25°C/90°C: setting at 25°C for 3 hr before cooking at 90°C for 15 min. Different letters in the same group of bars indicate a significant difference ($p < 0.05$).

$$\% \text{ Free water} = \frac{(\text{Total area (cm}^2) - \text{meat film area (cm}^2)) \times 9.47 \text{ mg water/cm}^2}{\text{Total moisture (mg) of gel sample}} \times 100\%$$

Sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE)

Preparation of samples for SDS-PAGE was performed by the methods of Morrissey et al. (1993) with slight modification. Sample (3g) were homogenized with 27 mL of 5% (w/v) sodium dodecylsulfate (SDS) solution for 2 min at a speed of 3–4 using a Polytron (Brinkmann Instruments, Westbury, NY). The homogenates were incubated in an 80°C water bath for 1 hr to dissolve all myofibrillar proteins and centrifuged at 8,000 × *g* for 20 min to remove undissolved debris which was mainly undissolved connective and fish skin. Electrophoresis was conducted according to the procedures of Laemmli (1970). Extracted proteins were boiled for 90 sec in a SDS-PAGE treatment buffer (1:1, v/v) and 40 µg protein, measured by the method of Lowry et al. (1951), was applied on a 4% stacking and 10% running polyacrylamide gel. A high molecular weight standard mixture (Stock No. SDS-6H, Sigma Chemical Company, St. Louis, MO) including myosin of rabbit muscle (205 kDa), β-galactosidase of *E. coli* (116 kDa), phosphorylase b of rabbit muscle (97 kDa), albumin of bovine (66 kDa), albumin of egg (45 kDa), and carbonic anhydrase of bovine erythrocytes (29 kDa) was used. Protein bands were stained with 0.125% Coomassie blue and destained in a 50% methanol and 10% acetic acid solution followed by a 5% methanol and 7% acetic acid solution. Quantitative analysis was performed using a laser scanning densitometer (Biomed Instruments, Inc., Fullerton, CA). The relative amount of myosin heavy chain in each sample was expressed as percentage compared to control surimi.

Analyses of proximate composition

Moisture, protein (total N × 6.25 − nonprotein nitrogen), fat, and ash were determined according to AOAC (1990) procedures. Nonprotein nitrogen was measured by the method of Woyewoda et al. (1986).

Chemical, microbiological, and enzymatic analyses

The quality of waste waters and the UF permeates were evaluated by measuring COD, %T, APC, and protease activity. The COD was measured using the procedures described by APHA (1980). Water clarity (absorbance) was monitored at 500 nm using a spectrophotometer (Model DU-640, Beckman Instruments, Inc., Fullerton, CA) and converted to %T. For APC, waste waters and filtrates were serially diluted in 1% peptone water. Total aerobes were enumerated, in duplicate, using 3M aerobic count plates (Petrifilm, Inc., St. Paul, MN). Plates were incubated at 32°C for 48 hr. Protease activity was assessed by the method of An et al. (1994a) using azocasein (Sigma Chem. Co., St. Louis, MO) as a substrate. Absorbance of the reaction mixture was measured at 450 nm by a spectrophotometer (Model DU-640, Beckman Instruments, Inc. Fullerton, CA). Results were expressed as A_{450}.

Statistical analysis

Statistical analysis of data was carried out using one way analysis of variance. Differences among mean values were established using the least significance difference (LSD) multiple range test (Steel and Torrie, 1980). Mean values were considered significant when $p < 0.05$.

RESULTS & DISCUSSION

Compositional profile of Pacific whiting surimi wastes

The amount of discharged waste and compositional properties of the waste water were compared (Table 1). In producing 1 kg of surimi, 29.1 ± 3.5L (mean ± S.D.) of waste water were discharged from the processing line (W1 through W6). The majority (≈75%) of the waste water was discharged from screeners (W3 and W5) while the waste water released from dehydrators (W1, W2, W4) and screw press (W6) comprised a relatively minor amount. The processing water discharged from the first dehydrator (W1) contained the highest level of protein, non-

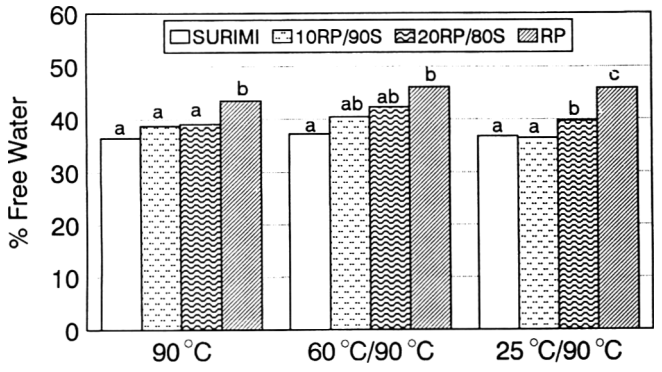


Fig. 6—Loss of expressible free water (%) of gels. S: regular surimi; RP: MF recovered protein; 10RP/90S: 10% RP and 90% S; 20RP/80S: 20% RP and 80% S; 90°C: cooked at 90°C for 15 min; 60°C/90°C: 60°C incubation for 30 min followed by cooking at 90°C for 15 min; 25°C/90°C: setting at 25°C for 3 hr before cooking at 90°C for 15 min. Different letters in the same group of bars indicate a significant difference ($p < 0.05$).

protein nitrogen, fat, and ash. The waste water in successive discharge points revealed decreasing protein, nonprotein nitrogen, fat, and ash. However, W1 constituted only 1.5% of the total processing water and had a strong fishy smell. The high protein content (Table 1), presence of low molecular weight protein bands (29–45 kDa), and insignificant amount of myosin heavy chain (Fig. 2 and 3) in the first waste water suggested that the first washing removed mainly sarcoplasmic proteins and did not impart notable losses of myosin. The removal of sarcoplasmic proteins is desirable for surimi processing since they interfere with heat-induced gelation of myofibrillar proteins (Yang and Froning, 1992). For this processing line, screen size 5 mm diameter was used for mincing fish muscle. Particle size of muscle was gradually reduced and sarcoplasmic proteins were gradually released from muscle compartments, dissolved in water, and removed from insoluble portions throughout successive washing and dewatering steps. This was evident from SDS-PAGE (Fig. 2) in which presence of low molecular weight bands occurred throughout the waste waters collected from different dewatering steps.

Results from SDS-PAGE (Fig. 2) showed that a considerable amount of myofibrillar proteins, myosin and actin, was lost in the waste water during washing and dewatering. Quantitative analysis, using a densitometer, showed that loss of myosin was small in the first waste water but increased substantially in the second waste water and then remained nearly constant throughout the rest of processing (Fig. 3). Generally the washing of minced fresh with water has been believed to remove sarcoplasmic proteins which in turn concentrates myofibrillar proteins. However, high losses of myofibrillar proteins during washing and dewatering were observed in our results. Extensive washing, which might cause myofibrillar proteins to be dissolved in water to some extent, and mechanical force during dewatering, which resulted in the loss of myofibrillar protein in the form of particulates, could be the major procedures responsible for the losses of the functional myofibrillar proteins in surimi processing. This confirmed the studies of Lin and Park (1994) in which the solubility of myofibrillar proteins increased as number of washing cycles increased. Since sarcoplasmic proteins and undesirable nonprotein nitrogen compounds, i.e., trimethylamine, dimethylamine, are fairly soluble in water, removal of these components could be accomplished in the first washing step by using enough water to solubilize remove them. Use of continuous washing cycles to remove all undesirable compounds and reduce losses of solids was suggested by Babbitt (1990).

Recovery of protein from surimi waste

When washed mince was subjected to the final dewatering, some protein, in the form of particulates, was lost through the orifice due to the mechanical force of the screw press. These insoluble particulates were recovered by a rotary screen and further concentrated by microfiltration (MF). The moisture of the discharged water (99.0%) was reduced to 93.3% by the rotary screen and then to 84.5% by the MF unit; the solids were increased by 6.7 fold and 15.5 fold, respectively. The solids in the MF recovered protein were concentrated to 16.3%. However, that was slightly lower than regular surimi (19.6%) (Table 2).

Table 4—Instrumental color values of the gels

(1) 90°C[e]	L*	a*	b*
Surimi (S)	80.92 ± 0.19[1a]	−3.77 ± 0.05[a]	4.43 ± 0.08[a]
10RP/90S	79.41 ± 0.29[b]	−3.73 ± 0.12[a]	3.88 ± 0.12[b]
20RP/80S	81.22 ± 0.19[c]	−3.64 ± 0.05[b]	4.25 ± 0.21[ab]
Recovered protein (RP)	81.62 ± 0.34[d]	−3.39 ± 0.06[c]	4.42 ± 0.24[a]
(2) 60°C/90°C[e]	**L***	**a***	**b***
Surimi (S)	81.40 ± 0.21[a]	−3.83 ± 0.07[a]	4.50 ± 0.25[a]
10RP/90S	80.45 ± 0.36[b]	−3.66 ± 0.06[b]	3.89 ± 0.29[b]
20RP/80S	82.04 ± 0.27[c]	−3.68 ± 0.06[b]	4.13 ± 0.14[c]
Recovered protein (RP)	82.65 ± 0.35[ac]	−3.39 ± 0.04[c]	4.99 ± 0.22[d]
(3) 25°C/90°C[e]	**L***	**a***	**b***
Surimi (S)	81.80 ± 0.23[a]	−3.75 ± 0.12[a]	4.43 ± 0.08[a]
10RP/90S	80.68 ± 0.29[b]	−3.72 ± 0.03[a]	4.02 ± 0.14[b]
20RP/80S	81.48 ± 0.19[a]	−3.65 ± 0.01[b]	3.91 ± 0.14[b]
Recovered protein (RP)	81.67 ± 0.57[a]	−3.43 ± 0.06[c]	4.45 ± 0.18[a]

[1] Values are mean ± S.D.

[abcd] Different letters in the same column within the same section (i.e., heating method) indicate significant difference.

[e] S: surimi; RP: MF recovered protein; 10RP/90S: 10% MF recovered protein with 90% surimi; 20RP/80S: 20% MF recovered protein with 80% regular surimi; (1) 90°C: heating at 90°C for 15 min; (2) 60°C/90°C: 60°C setting for 30 min followed by heating at 90°C for 15 min; (3) 25°C/90°C: 25°C setting for 3 hr followed by 90°C heating for 15 min.

In this process, the surimi production yield, amount produced per total whole whiting being processed, was 15.5 ± 1.5% (mean ± S.D.). For every 100 kg of surimi produced, 1.7 ± 0.2 (mean ± S.D.) kg protein was recovered by the MF unit. The surimi production yield could be increased by 1.7 ± 0.2% (by weight) and the change in total solids level would be insignificant if the MF recovered protein was added back to the regular processing line.

The low level of myosin in UF7 and the high myosin heavy content in RS (Fig. 3) indicated that most of the myosin lost in the form of particulate from the screw press was recovered by the rotary screen (Fig. 1). The myosin heavy chain in the protein recovered by rotary screen was subsequently increased by two- to threefold when microfiltration was applied. Due to high pressure created by microfiltration, some myosin was lost in the water portion (UF8) (Fig. 3).

Approximate composition of MF recovered proteins was compared with regular surimi sampled from the same processing line (Table 2). Results revealed that the MF recovered proteins contained less protein but higher ash than regular surimi ($p < 0.05$). No significant difference was observed in fat between regular surimi and MF recovered protein. As shown (Table 1), ash (minerals) was continuously lost during washing and dewatering. The higher ash in the MF recovered protein indicated that minerals lost in the wastes could be partially recovered by microfiltration (Table 2).

Microfiltration was not enough to recover proteins lost in waste water discharged from dehydrators (W1, W2, W4) or screeners (W3, W5) due to the fine particle sizes. However, moisture content in these streams was reduced to minimal level of 91.3% (Table 3) using ultrafiltration (UF) with a membrane of 30,000 molecular weight cut-off. Proteins concentrated by ultrafiltration had considerably darker color and strong odor. These results confirmed the work of French and Pedersen (1990) who observed that surimi block containing 10% UF concentrate and 90% regular surimi had a slight brownish hue and a distinct fishy odor.

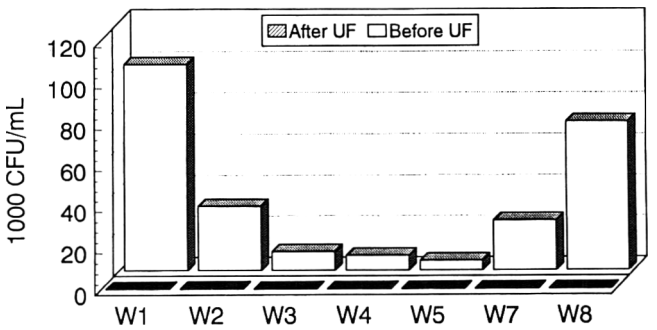


Fig. 7—Effect of ultrafiltration on aerobic plate count (APC) of surimi waste waters. W1 through W8 indicate different waste water streams as illustrated in Fig. 1.

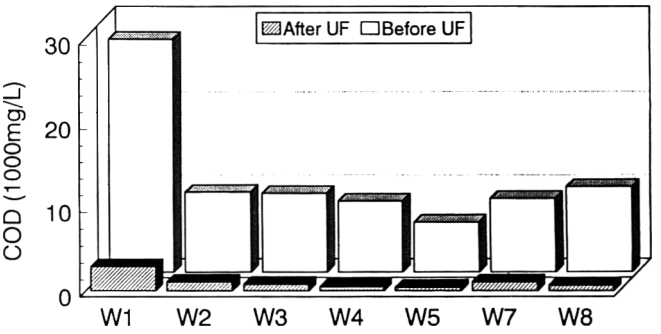


Fig. 8—Effect of ultrafiltration on chemical oxygen demand (COD) of surimi waste waters. W1 through W8 indicate different waste water streams as illustrated in Fig. 1.

Characterization of recovered protein

Gel functionality of recovered protein from MF was evaluated vs controls (regular surimi from the same process). Recovered protein revealed a lower stress but the same strain values as regular surimi, regardless of cooking method (Fig. 4 and 5). Gels made with 10% MF recovered protein and 90% regular surimi had no significant difference in stress or strain values compared to controls. When gels were made by replacing regular surimi with 20% MF recovered protein and preincubated at 25°C or 60°C, a significant reduction ($p < 0.05$) in stress value was noted. However, Korhonen and Lanier (1991) reported that substitution of regular surimi with 10% UF concentrate resulted in various degrees of weaker gels. The difference in gel quality between proteins recovered by MF and UF could be due to different levels of myosin (Fig. 2 and 3). The myosin heavy chain content in MF recovered proteins was five times higher than that of the UF concentrate. This was because the MF unit with screen sizes 50μ and 30μ, allowed some soluble proteins to pass through and concentrated the insoluble particulate which might have higher myosin.

Gels of MF recovered protein, regardless of cooking method, had a higher loss of free water than regular surimi gel when subjected to mechanical force (Fig. 6). However, by substituting 10% or 20% regular surimi with the recovered protein, the gel had the same expressible moisture as the regular surimi gel.

Gels of MF recovered protein, except those made by 25°C/90°C heating method, had better color, i.e., higher lightness (L*) and lower greenness (a*), than regular surimi gels (Table 4). When 10 or 20% of recovered protein was blended with regular surimi, the gels maintained better a* values and also showed lower b* values. This corresponded with results of Korhonen and Lanier (1991) showing that substitution of surimi with recovered proteins did not deteriorate surimi gel color. The effects of different cooking methods on gel color were not significant ($p < 0.05$). This also confirmed previous work of Park (1994).

Characterization of waste waters and UF permeates

APC was relatively high in the first discharged water and substantially reduced in successive waste water streams (Fig. 7). The increased APC in W7 and W8 were probably due to washing of the microbial load from the meat into the waste stream as a result of the elevated pressures created by the screw press and microfiltration. Regardless of point of sampling, APC levels in every UF permeate were insignificant (<100 CFU/mL).

The extensive washing of minced flesh caused a high loss of organic substances which resulted in extremely high COD (6,000 to 27,000 mg/L) in the waste waters (Fig. 8) compared to 900–1,200 mg/L from poultry processing waste (Chang and Sheldon, 1989b; Chang et al., 1989). However, the COD levels of the wastes were comparable to study of Swafford (1987) in which the COD values were 9,700 to 50,000 mg/L for surimi waste of Alaska pollock. This high level was especially noted in the first discharged waste (Fig. 8) in which organic substances were relatively high (Table 1). By applying UF, an 89–94% reduction in COD value was achieved. However, the UF permeate of the first waste water still had a high COD value.

The protease activity in waste waters did not decrease with successive washing processes (Fig. 9). The solubility of Pacific whiting proteases during washing was not complete. However, during postmortem, part of the whiting proteases might associate with myofibrils due to parasite effects (An et al., 1994b). This supports our observation that the waste water discharged from dehydrators and the screw press, in which some particulates were squeezed through the orifice into the water, had relatively high ($P < 0.05$) protease activity. Our results were in agreement with Seymour et al. (1994) in which hydrophobic characteristics of cathepsin L were reported. Cathepsin L, a primary protease, could associate with myofibrils through hydrophobic interaction making it less soluble in water. Thus, whiting proteases were only partially removed by each washing and dewatering step.

Our observation confirmed that whiting protease was highly associated with protein particles. With application of UF, using a membrane of 30,000 MWCO to recondition water, a substantial reduction of protease activity in permeate was noted (Fig. 9). These results suggest that whiting protease (with 28 kDa, Seymour et al., 1994), due to its high affinity with protein, could be separated from water with a higher molecular weight cut-off membrane.

Clarity of waste water increased in successive discharge points (Fig. 10) but decreased in waste water from the screw press through which more solids were liberated into the waste water. The clarity of surimi wastes (0.35 to 25.57% T) were relatively lower than that of poultry chiller water (13 to 27% T, Chang and Sheldon, 1989a). However, UF permeates of all surimi waste waters were very clear with average percent transmittance 97.8. This was nearly identical to that of the tap water (98.4%T) used for processing. The clarity of UF permeates indicated the potential for water recycling in surimi operation.

CONCLUSIONS

THE PROTEIN, in the form of particulates, lost in the final dewatering process (screw press) could be successfully recovered and concentrated using a rotary screen and microfiltration. Surimi with 10% replacement of MF recovered protein had the same gel quality as regular surimi with respect to gel hardness, elasticity, water retention, and color. The gel made with MF recovered protein had better color quality than regular surimi gels. The surimi production rate could be increased by 1.7% by adding the MF recovered protein back to the production line without diminishing surimi functionality. The APC, COD, turbidity, and protease levels of waste waters were substantially reduced by ultrafiltration. Use of UF equipment provides the possibility of recycling processing water in the leaching system. However, proteins concentrated by ultrafiltration had considerable dark colors and strong odors.

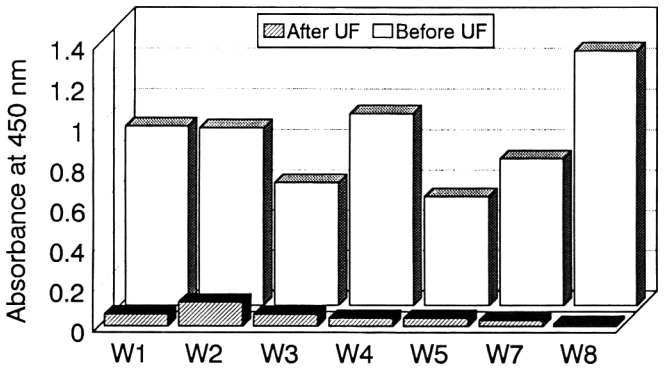


Fig. 9—Effect of ultrafiltration on protease activity of surimi waste waters. W1 through W8 indicate different waste water streams as illustrated in Fig. 1.

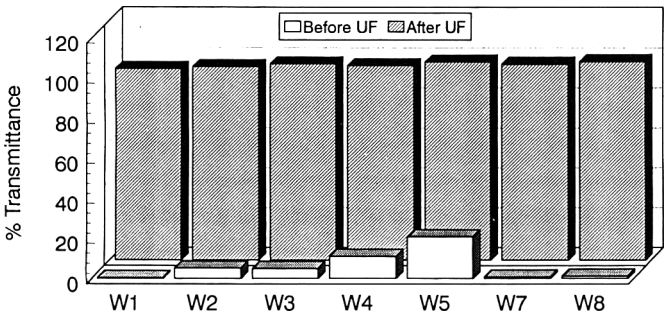


Fig. 10—Effect of ultrafiltration on clarity (%T) of surimi waste waters. W1 through W8 indicate different waste water streams as illustrated in Fig. 1.

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Ohmic Heating Maximizes Gel Functionality of Pacific Whiting Surimi

J. YONGSAWATDIGUL, J. W. PARK, E. KOLBE, Y. ABU DAGGA, and M. T. MORRISSEY

ABSTRACT

Surimi without enzyme inhibitors containing 78% moisture and 2% NaCl was heated conventionally and ohmically to 90°C after holding at 55°C for 0, 1, 3 and 5 min. Gels heated slowly in a water bath exhibited poor gel quality, while the ohmically heated gels without holding at 55°C showed more than a twofold increase in shear stress and shear strain over conventionally heated gels. Degradation of myosin and actin was minimized by ohmic heating, resulting in a continuous network structure. Ohmic heating with a rapid heating rate was an effective method for maximizing gel functionality of Pacific whiting surimi without enzyme inhibitors.

Key Words: ohmic heating, surimi, proteolysis, whiting

INTRODUCTION

PACIFIC WHITING (*Merluccius productus*) is the most abundant fishery resource off the West Coast of the U.S. with an allowable biological catch of 140,000 to 250,000 metric tons for the last three years (NMFS, 1993). The major portion of the harvested fish is used to produce surimi, a washed and dewatered fish mince, which is further utilized as a raw ingredient for surimi seafood products (Pacheco-Aguilar et al., 1989). Due to the presence of heat-stable endogenous protease in the flesh (Nagahisa et al., 1981; Patashnik et al., 1982; Erickson et al., 1983), Pacific whiting surimi normally exhibits undesirable textural properties unless food grade enzyme inhibitors are added (Chang-Lee et al., 1989; Morrissey et al., 1993). The protease responsible for textural degradation was purified and characterized as cathepsin L with an optimum temperature at 55°C (Seymour et al., 1994). A gel weakening at around 55–60°C has also been reported in threadfin bream (*Nemipterus bathybius*) (Toyohara and Shimizu, 1988), Atlantic menhaden (*Brevoorti tyrannus*) (Boye and Lanier, 1988), arrowtooth flounder (*Atheresthes stomias*) (Greene and Babbitt, 1990), white croaker (*Micropogon opercularis*) and oval filefish (*Navodon modestus*) (Toyohara et al., 1990).

Beef plasma proteins, egg white, and other food grade enzyme inhibitors have been used to inhibit proteolytic activity (Nagahisa et al., 1981; Chang-Lee et al., 1989; Hamann et al., 1990; Porter, 1992; Morrissey et al., 1993). However, these enzyme inhibitors used commercially have some negative effects such as high cost, off-odor, off-color and labeling concerns. Consequently, processing alternatives to overcome the textural deterioration of Pacific whiting surimi are needed.

Ohmic heating is a method in which alternating electrical current is passed through an electrically conducting food product (Biss et al., 1989). Heat is internally generated, resulting in a rapid heating rate. Because heat is simultaneously generated in liquid and solid phases, the temperature increase in the product is uniform, compared with the conventional process in which heat is applied at the external boundary (de Alwis and Fryer, 1990; Parrot, 1992).

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To minimize proteolysis, thermal inactivation of the enzyme should be quickly achieved (Patashnik et al., 1982). Greene and Babbitt (1990) demonstrated that textural degradation caused by proteolysis in arrowtooth flounder was notably reduced when cooked by microwaves. However, microwave processes can create non-uniform heating patterns because of differences in dielectric properties of the sample and nonuniform microwave field distribution (Decareau, 1985). In addition, microwave heating rates are so rapid that conductive heat transfer cannot uniformly take place (Datta and Hu, 1992). Since ohmic heating provides a rapid heating rate with more uniform temperature distribution than microwave heating, it could be an alternative to overcome the protease problem. Our objective was to investigate the feasibility of ohmic heating to maximize gel functionality of whiting surimi without enzyme inhibitors.

MATERIALS & METHODS

UNFROZEN PACIFIC WHITING (*Merluccius productus*) surimi (100 kg) without food grade enzyme inhibitors and cryoprotectants was obtained from Point Adams Packing Co. (Hammond, OR). Surimi was packed in polystyrene foam containers with ice and delivered to the OSU Seafood Laboratory (Astoria, OR) within 30 min. Each 17 kg of surimi was mixed with cryoprotectants, 4% sucrose, 4% sorbitol (ICI Specialties, New Castle, DE), and 0.3% sodium tripolyphosphate (B.K. Ladenburg Corp., Cresskill, NJ) using a Hobart vertical mixer (Hobart Manufacturing Co., Troy, OH) for 2 min. Mixing was carried out in a cold room (3°C). Mixed surimi was then divided into ≈ 500-g portions and vacuum-packed in plastic trays. Samples were frozen and stored in a −30°C room.

Surimi gel preparation

Frozen surimi samples were thawed at room temperature (≈23°C) for 2 hr and cut into small pieces. Moisture content of surimi was determined using the microwave procedure described by Morrissey et al. (1993). One kg of surimi was chopped for 1.5 min in a Stephan vertical vacuum cutter (model UM 5 universal, Stephan Machinery Co., Columbus, OH) at low speed. Salt was added and mixed with surimi another 1.5 min. Then, ice was added to adjust final moisture to 78%, and the sample was further chopped at high speed under vacuum of 600 mm Hg for 3 min. The paste was maintained below 8°C during chopping. An aliquot of the paste (≈300g) was stuffed into stainless steel cooking tubes (1.9 cm i.d. × 17.5 cm long) and heated in a 90°C water bath for 15 min. Change in temperature at the geometric center of the tube sample

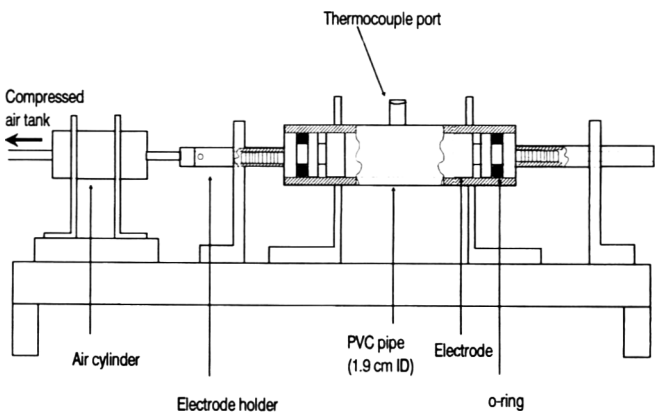


Fig. 1—Sample tube and electrodes.

was measured using a T-type thermocouple, and recorded every 30 sec in a datalogger (model 21x, Campbell Scientific Inc., Logan, UT). The remainder was stuffed into PVC tubes (1.9 cm i.d. × 20.5 cm long) and heated using an ohmic heating apparatus. Surimi gels heated by both heating methods were kept at 4°C for torsion tests, scanning electron microscopy, and gel electrophoresis within 24 hr. Gel preparation was replicated twice.

Ohmic heating apparatus

An ohmic heating apparatus was developed from two rhodium-coated stainless steel electrodes at each end of a PVC tube (1.9 cm i.d. × 20.5 cm long), a variable transformer (Powerstat F246, Newark Electronics, Eugene, OR), a current transducer (CT5-010A, Syntex Co., Wilsonville, OR), and a voltage transducer (VT-240A, Syntex Co., Wilsonville, OR). To minimize electrical hazards, the sample tube and electrodes were housed inside a plexiglass enclosure equipped with an open-snap action switch, which was activated when the plexiglass box was closed.

A 1.6-mm-diameter hole was drilled at the center of the PVC tube, and a saddle (diameter = 1.9 cm) was glued in that position to make a thermocouple port. To measure temperature at the geometric center of the sample, a 30-gauge Teflon-sealed type-T thermocouple (OMEGA Engineering Inc., Stamford, CT) with a compression fitting (OMEGA Engineering Inc., Stamford, CT) was used. Sixty-Hz alternating current at 240 V was supplied to a variable transformer. Voltage output ranged from 0 to 280 V. Voltage and current transducers were used to measure voltage across and current through the sample, respectively.

Ohmic heating procedure

A sample tube containing surimi paste was placed on the sample holders (Fig. 1). An electrode was inserted into each end of the tube to obtain a sample length of ≈15 cm. One of the electrode support rods was connected to an air cylinder. Pressure of 448 kPa was applied to the sample to maintain an air-free contact between the electrodes and the paste. Based on preliminary studies, gel strength of surimi and corrosion of the electrodes increased with applied voltage gradient. However, at voltage gradient >13.3 V/cm, corrosion was so severe that it interrupted electrical circuits. Therefore, the apparatus was operated at the voltage gradient of 13.3 V/cm (applied voltage of 200 V). The pastes were heated to 55°C, and were held at 55°C for 0, 1, 3, and 5 min. Temperature was controlled by manually adjusting the variable transformer. After each holding time was achieved, the sample was heated to 90°C by re-applying 200 V. Each heating treatment was replicated 3 times. Temperature, voltage, and current changes during heating were recorded at 1 sec intervals on a datalogger (model 21X, Campbell Scientific, Inc., Logan, UT).

Torsion test

Torsion failure tests were performed as described by NFI (1991). Gels were equilibrated to and tested at room temperature. Ten hourglass samples were used for each treatment. Shear stress and shear strain at failure were calculated from equations described by Hamann (1983).

Sodium dodecyl sulfate-gel electrophoresis (SDS-PAGE)

Unheated surimi paste and heated samples were analyzed by SDS-PAGE. Twenty seven mL of heated (95°C) 5% sodium dodecyl sulfate solution was added to 3g sample. The mixtures were homogenized for 1 min at a speed setting of 3–4 by a Polytron (Brinkmann Instruments, Westbury, NY). The homogenates were incubated in an 80°C water bath for 1 hr, and were centrifuged at 7,000 × *g* (Sorvall, DuPont Co., Newton, CT) for 10 min at room temperature. The protein concentration of supernatants was measured by the Lowry method (Lowry et al., 1951), using bovine serum albumin (Sigma Chemical Co., St. Louis, MO) as a standard.

Electrophoresis was carried out according to the procedure of Laemmli (1970). Stacking gels and separating gels were 4% (w/v) and 10% (w/v) polyacrylamide, respectively. The amount of protein loaded on the polyacrylamide gel was 60 µg. The separated proteins were stained with 0.125% Coomassie brilliant blue R-250 (Bio-Rad, Richmond, CA), and destained in a solution containing 25% ethanol and 10% acetic acid.

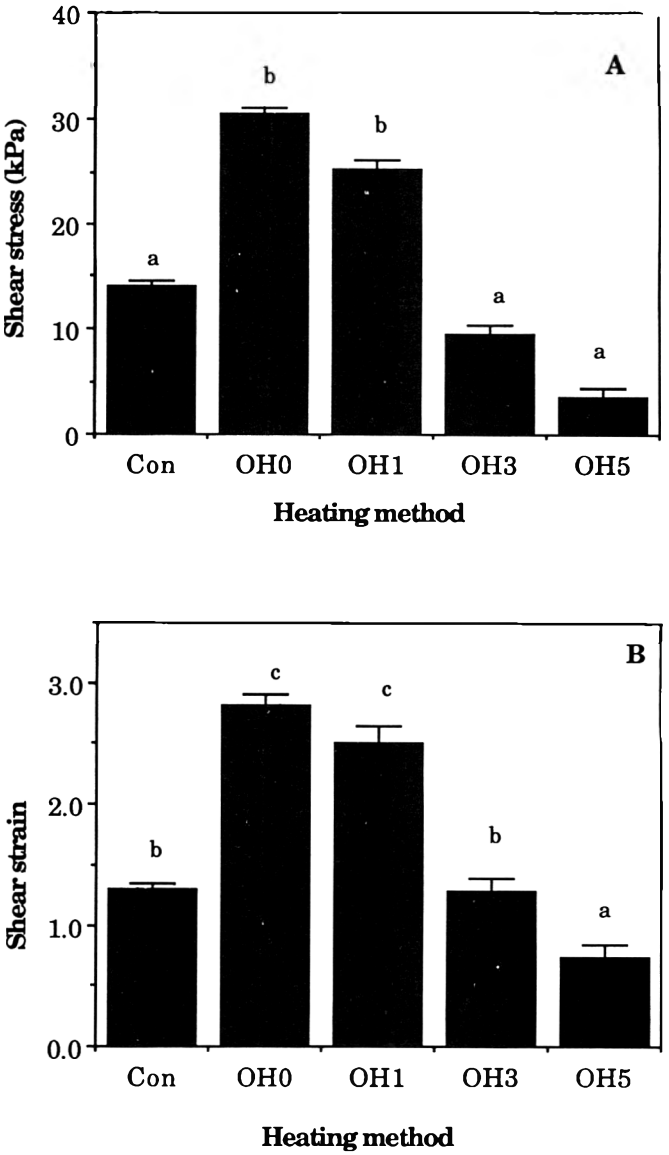


Fig. 2—Shear stress (A) and strain (B) of surimi gels heated by various heating methods. Means with the same letter (a,b,c) are not significantly different (P>0.05). CON = conventional heating (heated in a 90°C water bath for 15 min). OH0 = heated ohmically with voltage gradient of 13.3 V/cm (applied voltage = 200V) to reach 90°C. OH1 = heated ohmically with voltage gradient of 13.3 V/cm and held for 1 min at 55°C before heating to 90°C. OH3 = heated ohmically with voltage gradient of 13.3 V/cm and held for 3 min at 55°C before heating to 90°C. OH5 = heated ohmically with voltage gradient of 13.3 V/cm and held for 5 min at 55°C before heating to 90°C

Scanning electron microscopy

Small samples of heated gels were cut with a razor blade and fixed for 2 hr in 1% osmium tetroxide solution buffered with 0.125 cacodylate buffer pH 7.2. Samples were immersed for 20 min in 1,1-dimethoxypropane to remove unreacted osmium tetroxide and water. They were then soaked in absolute acetone twice, (10 min each). The samples were freeze-dried (model 500, Refrigeration for Science Inc., Island Park, NY) at a vacuum of 750 mm Hg and −73°C for 6 hr. Dried samples were mounted on aluminum specimen stubs (Ted Pella Inc., Redding, CA) using DUCO cement (Devcon Corp., Wood Dale, IL), and coated with gold in a sputter (model S150B. Edwards High Vacuum, West Sussex, England) for 45 sec at 10 kV and 40 mA. The samples were examined at 7,500× using a scanning electron microscope (Amray 1000A, Bedford, MA) at an accelerating voltage of 10 kV.

Statistical analysis

Since there were no significant differences (P > 0.05) between two replications, data were evaluated by one-way analysis of variance using

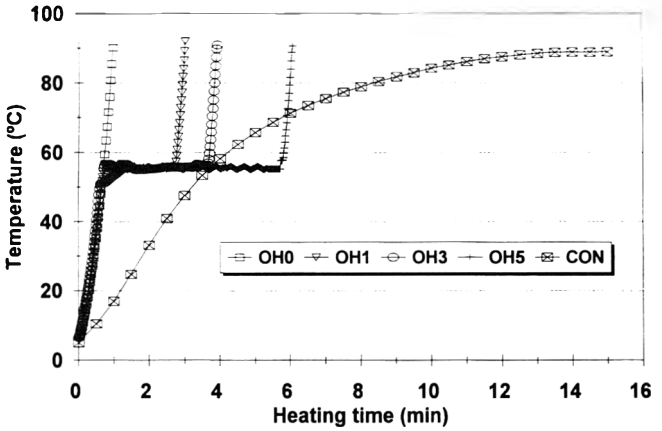


Fig. 3—Temperature profiles of surimi gels heated by various heating methods (abbreviations indicated in Fig. 2).

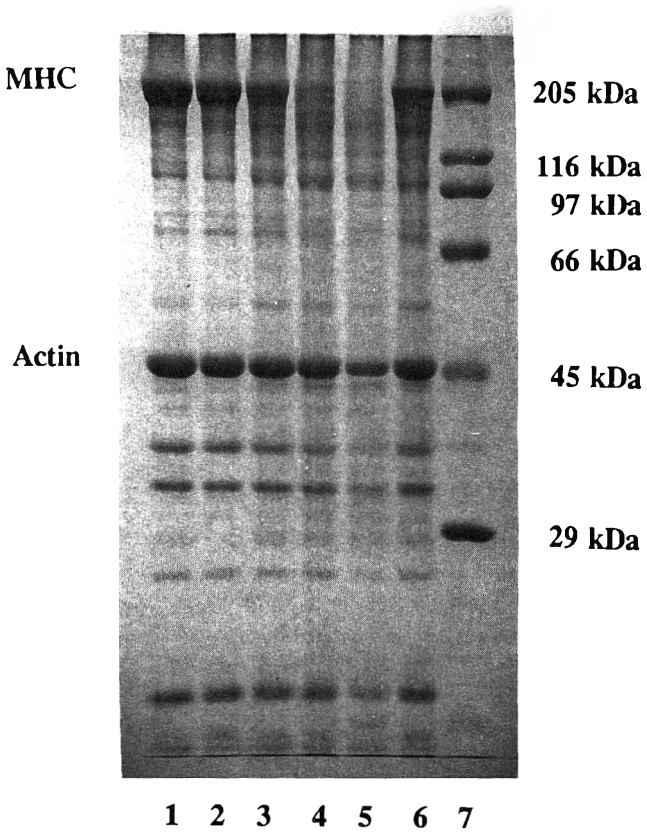


Fig. 4—SDS-PAGE separation pattern of surimi gels heated by various heating methods: (1) unheated surimi paste; (2) surimi heated ohmically with 13.3 V/cm to 90°C; (3) surimi heated ohmically with 13.3 V/cm and held at 55°C for 1 min before heating to 90°C; (4) surimi heated ohmically with 13.3 V/cm and held at 55°C for 3 min before heating to 90°C; (5) surimi heated ohmically with 13.3 V/cm and held at 55°C for 5 min before heating to 90°C; (6) surimi heated conventionally; (7) High molecular weight standard; MHC: Myosin heavy chain.

STATGRAPHIC Version 6.0 (Manugistics Inc., Rockville, MD). Differences within heating methods were determined using the least significant difference (LSD) multiple range test (Box et al., 1978).

RESULTS & DISCUSSION

Effects of heating methods on gel strength and myosin heavy chain

Shear stress and shear strain values of surimi gels heated conventionally (CON) were compared with those heated ohmically (Fig. 2A and 2B). Shear stress and shear strain are correlated with surimi gel hardness and cohesiveness, respectively (Hamann and MacDonald, 1992). Hardness of OH0 and OH1 gels was significantly higher than that of CON, OH3, and OH5 gels ($P<0.05$). No significant differences occurred in cohesiveness between CON and OH3 gels ($P>0.05$). However, cohesiveness of these two gels was significantly lower than that of OH0 and OH1, and higher than that of OH5 gels ($P<0.05$). Since an acceptable surimi gel should have a strain value of at least 2.0 (Hamann et al., 1990), textural properties of surimi gels heated in a 90°C water bath and those held ohmically at 55°C for 3 and 5 min were very poor. Low shear stress and shear strain in OH3 and OH5 illustrated the effect of protease activity at 55°C on textural characteristics of Pacific whiting surimi gel. Those gels heated ohmically to 90°C with a voltage gradient of 13.3 V/cm (200 V) and those held in ohmic heating at 55°C for 1 min showed good textural properties with strain values of 2.81 and 2.50, respectively. These were more than twice the value of conventionally heated surimi gels. Based on torsion test results, Pacific whiting gels without enzyme inhibitors heated ohmically without holding at 55°C were slightly better than those heated conventionally with incorporation of enzyme inhibitors such as beef plasma proteins, egg white, and potato extract. These had a maximum strain value around 2.5 (Morrissey et al., 1993).

Seymour et al. (1994) illustrated that activity of the protease purified from Pacific whiting gradually increased as temperature increased until peaking at 55°C. It then started decreasing and reached a minimum at 70°C. The myosin and actin have been reported as a substrate of endogenous protease found in threadfin-bream surimi (Toyohara and Shimizu, 1988; Toyohara et al., 1990), and of cathepsin L purified from rabbit skeletal muscle (Okitani et al., 1980) and Pacific whiting (An et al., 1994). Temperature profiles of conventional heating (Fig. 3), indicate that surimi was slowly heated in a 90°C water bath and was exposed to a temperature range of 40–60°C for ≈2 min. Moreover, it took about 6 min to reach 70°C at which the protease could be inactivated. Due to the slow heating rate, the enzyme was activated and started degrading the myosin before thermal inactivation occurred. Since the myosin plays an important role in gel network formation (Niwa, 1992), severe degradation of the myosin heavy chain (Fig. 4) resulted in low gel strength (Fig. 2A and 2B). When holding time at 55°C in ohmic heating was prolonged, integrity of the myosin heavy chain and actin were diminished (Fig. 4). The OH0 and OH1 samples exhibited higher intensity of myosin and actin than the CON sample, while less intensity was observed in the OH3 and CH5. As surimi was subjected to optimum temperature of the enzyme for a longer period of time, more degradation of myosin and actin occurred. For the sample held at 55°C for 5 min, the myosin heavy chain and actin almost disappeared. These results suggest that gel-weakening associated with breakdown of the myosin heavy chain and actin in Pacific whiting surimi was due to proteolytic activity. The highest gel strength and the most intense myosin heavy chain and actin bands were observed in OH0 gels that were heated to 90°C within 1 min. The myosin and actin bands of the OH0 were comparable to those of unheated surimi paste (Fig. 4). Probably rapid thermal inactivation minimized degradation of the myosin and actin.

The effect of ohmic heating on surimi gel quality was also reported by Shiba (1992). Gel strength of surimi made from walleye pollock, white croaker, and sardine was increased when ohmically heated at a heating rate of 47°C/min was applied. Shiba and Numakura (1992) illustrated that walleye pollock surimi (diameter = 3 cm, length = 18 cm) heated by ohmic heating within 1 min exhibited a 33.1% increase in gel strength and a 38.1% increase in myosin heavy chain content in comparison with that heated conventionally 50 min. Gel strength and the myosin heavy chain content were decreased when the samples were heated ohmically with a slow heating rate. Since there is no substantial evidence of the presence of endogenous prote-

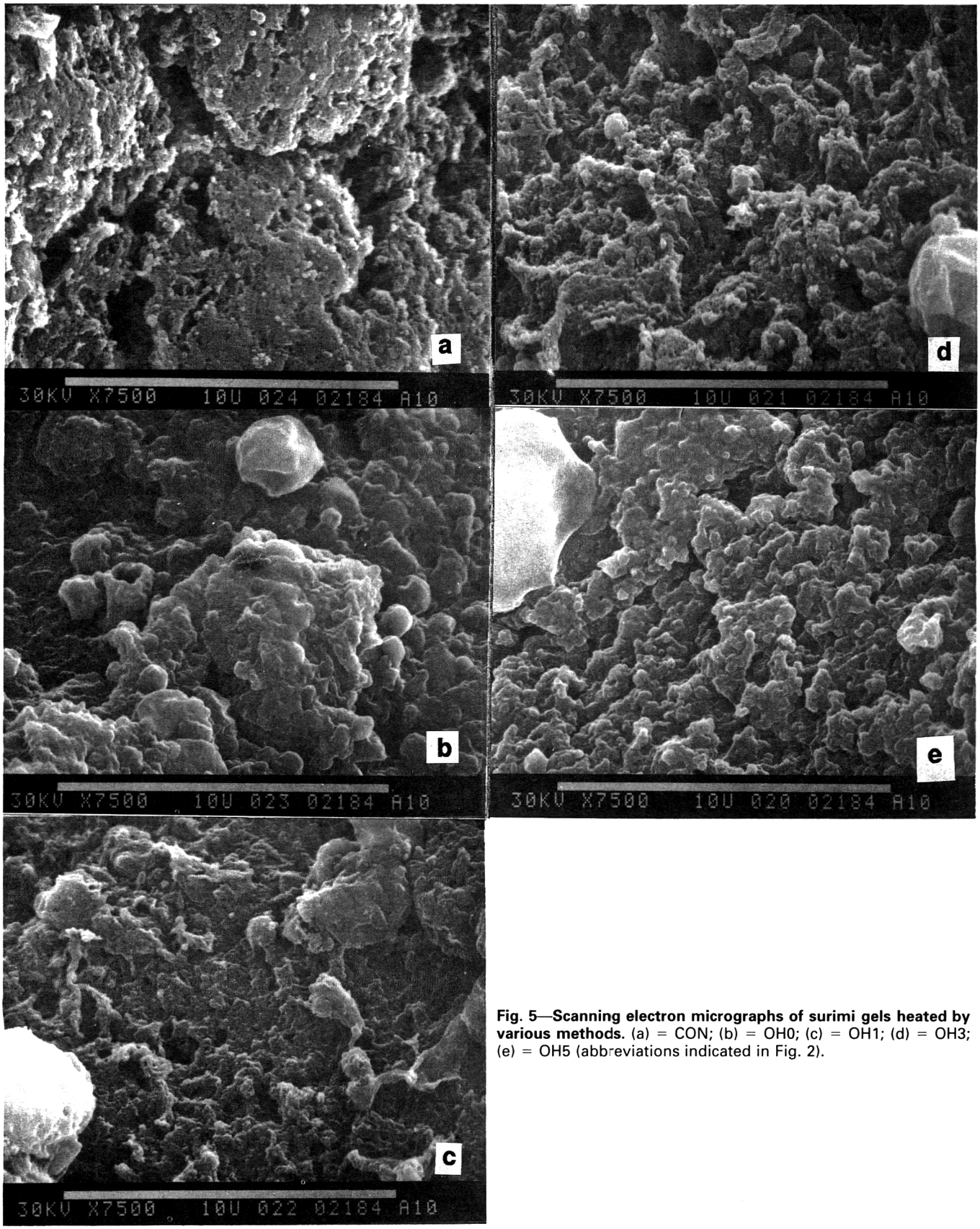


Fig. 5—Scanning electron micrographs of surimi gels heated by various methods. (a) = CON; (b) = OH0; (c) = OH1; (d) = OH3; (e) = OH5 (abbreviations indicated in Fig. 2).

ase(s) in walleye pollock, an increased gel quality may not be solely affected by rapid inactivation of the protease(s). However, ohmic heating with a rapid heating rate can evidently improve surimi gel quality.

Although the intensity of the myosin heavy chain band of CON gels was visually greater than that of OH3 gels, no differences ($P > 0.05$) occurred in shear strain and shear stress between those two gels. Toyohara and Shimizu (1988) also found a similar discrepancy. They indicated that the intensity of myosin did not accordingly correlate with gel strength. We could hypothesize that, once the myosin was degraded by the enzyme to a specific level, the gel network could not be properly

formed regardless of degree of degradation. This would agree with the fact that intact myosin is required for gel formation (Ziegler and Acton, 1984). When the myosin is severely hydrolyzed to smaller protein fragments (OH3 and OH5 in Fig. 4), a gel network was hardly developed, resulting in extremely low gel strength and very mushy texture.

Effects of heating methods on gel microstructure

Scanning electron micrographs of surimi gels heated by various heating methods were compared (Fig. 5). Microstructure of CON gels showed a sponge-like structure with several voids (Fig. 5a). The protein particles appeared to be randomly aggregated, without ordered network formation. This corresponded to a poor gel strength observed in the conventionally heated samples. On the other hand, OH0 gels involved formation of a continuous network exhibiting some definite degree of order (Fig. 5b). Microstructures of ohmically heated gels showed more openings and discontinuity as holding time at 55°C increased (Fig. 5c, 5d, 5e), particularly the samples held at 55°C for 5 min. These micrographs suggested that poor gel network formation resulted from degradation of myofibrillar proteins caused by proteolysis.

CONCLUSIONS

PACIFIC WHITING GELS rapidly heated to 90°C by ohmic heating with a voltage gradient of 13.3 V/cm demonstrated superior shear stress and shear strain. Degradation of the myosin heavy chain and actin was significantly minimized. Scanning electron micrographs showed a continuous gel structure corresponding to its gel strength. A decreased gel strength associated with poor gel network and degradation of the myosin heavy chain and actin was noticed as holding time at optimum temperature of the protease, 55°C, was prolonged. Surimi gels were susceptible to proteolysis under conventional heating since a slow heating rate allowed the enzyme to degrade more myosin heavy chain and actin.

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Surimi Gel Colors as Affected by Moisture Content and Physical Conditions

JAE W. PARK

ABSTRACT

Colors (CIE L*, a*, and b*) of Alaska pollock (*Theragra chalcogramma*) and Pacific whiting (*Merluccius productus*) surimi gels were evaluated and related to compositional and physical conditions during preparation and measurements. Ten samples were used in each treatment. Water addition, sample size, species, cooking and test temperatures, and freeze/thaw affected lightness (L*) and yellowness (b*) values, while outside light conditions at measurement did not affect colors. Greenness (−a*) values were less affected by all conditions.

Key Words: color, surimi gel, moisture, temperatures

INTRODUCTION

SURIMI PROCESSING requires extensive washing to remove fat and other undesirable materials like pigments. Thus, raw surimi is naturally mild in odor and translucent in appearance. Myoglobin and hemoglobin, responsible for the red hue of fish meat, are thoroughly removed during the dewatering step of surimi processing. However, commercial surimi has four to five different grades based on the L* and b* values of cooked gels (Park and Morrissey, 1994). Surimi gels are generally opaque to translucent. Color, like texture and flavor, is an important quality aspect of surimi and it's related products. Many different commercial surimi seafoods have unique color and appearance based on its gel and applied pigments. Park and Morrissey (1994) reported that, for measuring its color, the world surimi industry uses three instruments: Hunter, Minolta, and Nippon Denshoku. All are tristimulus filter colorimeters based on the same technological principles. However, the instruments do not provide the similar color values for a given sample. Measurements vary between instruments based on their ability to consistently match light source-filter-detector responses. However, repetitive results can be obtained as long as a specified instrument is used.

Surimi processing technology, in the 1970's and 1980's has developed innovations for different types of surimi seafood with great emphasis on textural/rheological characteristics. Colors of surimi gels were occasionally reported as a part of experiments. However, little effort has been reported on extensive studies of the colors of surimi gels. Gel color is an important characteristic of surimi seafood. Our objective was to investigate the effects of moisture contents and physical conditions on gel colors of Alaska pollock and Pacific whiting surimi.

MATERIALS & METHODS

Sample preparation

Alaska pollock (*Theragra chalcogramma*) and Pacific whiting (*Merluccius productus*) surimi were obtained from local manufacturers. Both types were less than 6 mo old and commercial high grade (FA). Surimi was cut into 1,000-g blocks and kept in a freezer (−35°C) during the experiments. The moisture content of both surimi types was similar (75.2% for pollock and 74.5% for whiting). Both surimi contained 4% sugar, 4% sorbitol, and 0.3% sodium tripolyphosphate as cryoprotectants. However, in Pacific whiting surimi, 1.2% beef plasma protein was additionally included as an enzyme inhibitor.

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For the experimental plan (Fig. 1) each of the two types of surimi was divided into 4 groups with different levels of water addition: 0, 10, 20, and 30%. Based on moisture content and water addition, the final moisture content of each treatment was calculated: 73.6, 76.1, 78.6, and 81.1%, respectively. Each surimi paste was prepared as follows: Frozen surimi was thawed at room temperature for 2 hr and cut into 3-cm cubes. Surimi cubes were placed in a Stephan vacuum cutter (Stephan Machinery, Columbus, OH). For the first 1.5 min, frozen cubes were chopped at low speed. Salt (2%) was sprinkled in and chopping continued at low speed for 1 min. Then ice/water was added and chopped at low speed for 0.5 min. For the final 3 min, chopping was continued at high speed with vacuum (0.5 bar). During chopping, a constant cold temperature (<8°C) was maintained using a NesLab chiller (NesLab, Portsmouth, NH). The paste was stuffed into sausage casings (i.d. 3 cm) and stainless steel tubes (i.d. 1.9 cm) using a sausage stuffer (Sausage Maker, Buffalo, NY).

Each group was divided into setting and nonsetting treatments. For a setting treatment, samples were set at room temperature (25°C) for 3 hr prior to heating at 90°C for 30 min. Nonset samples were placed in hot water (~95°C) and the water temperature was immediately adjusted to 90°C using ice. Samples were heated for 30 min. Cooked gels were chilled rapidly in ice water. Gels were stored in a refrigerator until further treatments.

Color measurement

A CIE Lab color scale was used to measure the degree of lightness (L*), redness (+a*) or greenness (−a*), and yellowness (+b*) or blueness (−b*) of gels using a Minolta Chroma Meter CR-310 (Minolta Corp., Ramsey, NJ). The instrument was standardized using a Minolta calibration plate (Y_{CIE} = 94.5, x_{CIE} = 0.3160, y_{CIE} = 0.330) and a Hunter Lab standard hitching tile (L* = 82.13, a* = −5.24, b* = −0.55) with D_{65} illuminant and 2° observer. Ten gels were used for the measurement of each treatment. Gels, prepared as described above, were divided into three groups (Fig. 1):

(1) Gels were measured in the presence and the absence of fluorescent light to investigate effects of room light conditions.
(2) Gels were measured when the temperature of samples were 5°C and 25°C to investigate the effects of gel temperatures.
(3) Gels abused by freezing and thawing (0, 1, 3, 6, 9 cycles) were measured at room temperature under fluorescent light to investigate effects of frozen storage.

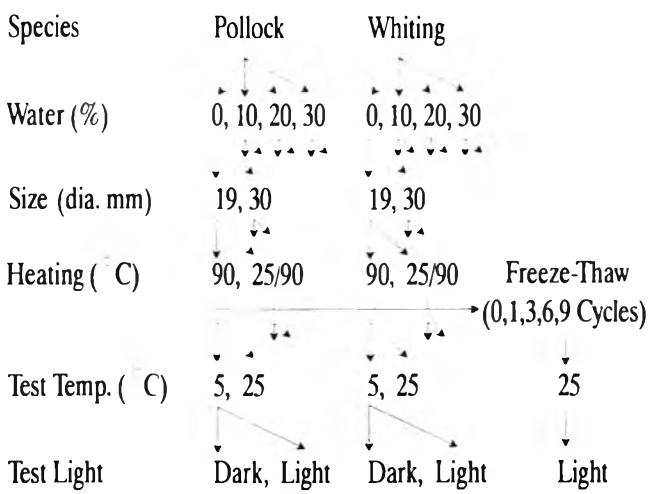


Fig. 1—Experimental design.

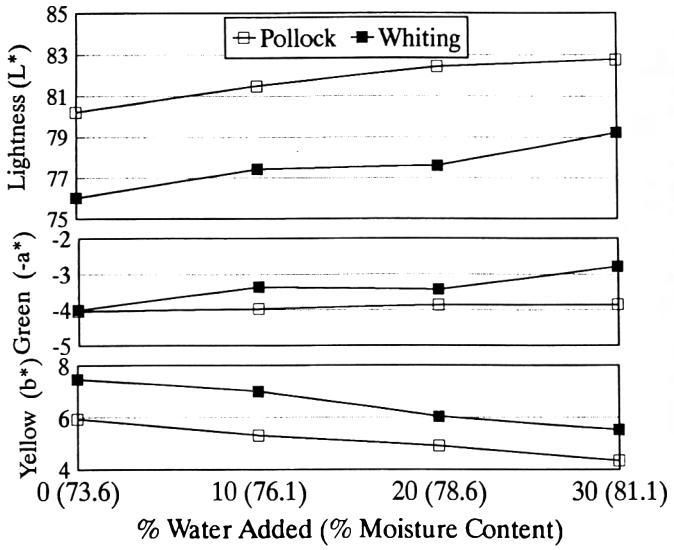


Fig. 2—Relation of species to gel colors with various levels of water addition. Whiting surimi contained 1.2% beef plasma protein.

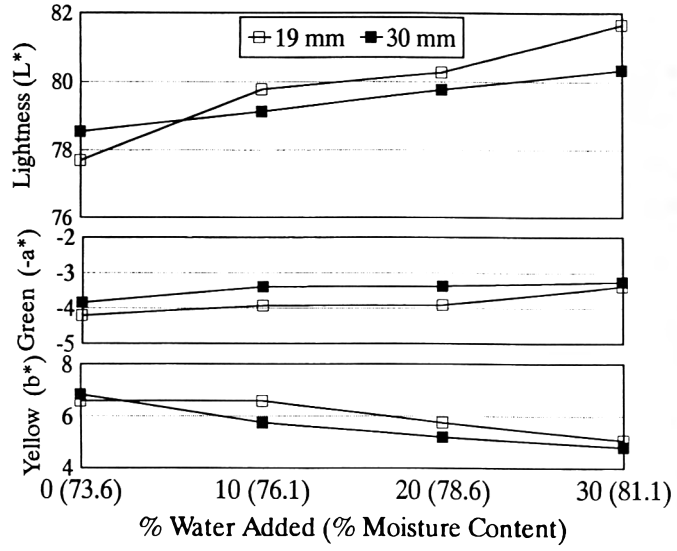


Fig. 3—Effects of sample sizes (diameter) on gel colors with levels of water addition.

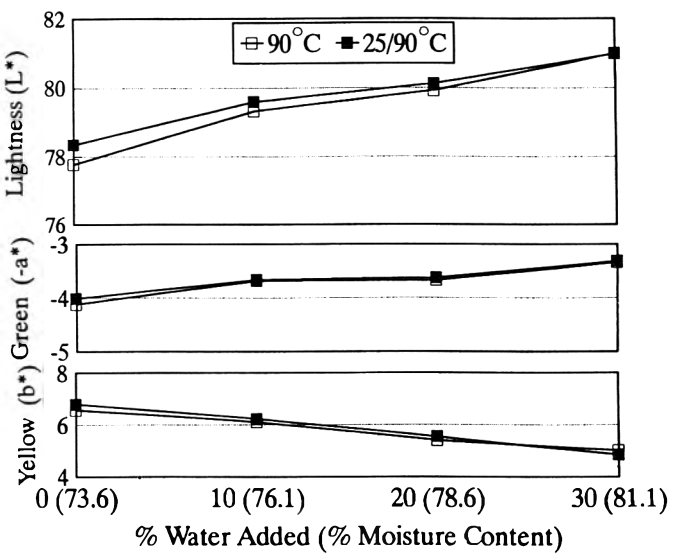


Fig. 4—Effects of heating temperatures on gel colors with levels of water addition. 90°C: heating at 90°C for 15 min. 25°/90°C: 3 hr setting at 25°C for 3 hr prior to heating at 90°C for 15 min.

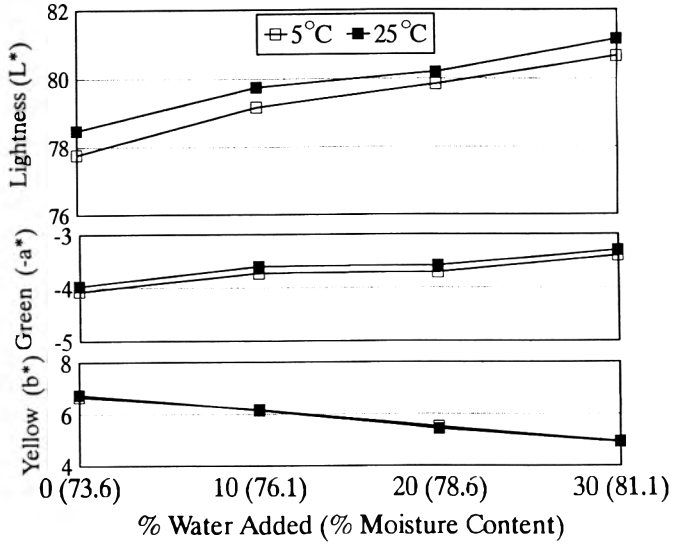


Fig. 5—Effects of gel temperatures on gel colors with levels of water addition.

After storing frozen (−18°C), gels were thawed at room temperatures (~2 hr) and the cycle was repeated 0, 1, 3, 6, 9 times.

Statistical analysis

Ten measurements of L*. a* and b* from each treatment were randomly divided into two groups. Averages of all response variables from each group were calculated. This provided two pseudo-replications for each treatment. The main effects and two-order interaction effects of response variables were analyzed using ANOVA. The least significant difference (LSD) was used to compare treatment means at $P < 0.05$. All statistical analyses were achieved using Statgraphics (Manugistics, Rockville, MD).

RESULT & DISCUSSION

Effects of moisture contents and species

Water addition affected L* and b* values ($P < 0.05$) (Fig. 2). The higher the moisture, the lighter and the less yellow the color. Gels with higher moisture contents looked whiter. This corresponded well on the whiteness equation (L − 3b*) recommended by Park (1994). With addition of 30% water, L* values increased from ~76 to ~79 for whiting surimi and ~80 to ~83 for pollock surimi. The change of green hue (−a*) was very small with increased water addition, though the green hue decreased in a linear response to water addition (Fig. 2). Yellow hue was reduced from ~7.5 to ~5.5 for whiting surimi and ~6 to ~4.5 for pollock surimi. A difference ($P < 0.05$) occurred in gel colors between Alaska pollock surimi and Pacific whiting surimi. Pacific whiting surimi showed 4 units less in L* values and 1.5 units more in b* values than Alaska pollock surimi, regardless of water addition. This was probably due to the beef plasma protein in the whiting surimi. Park (1994) also reported a decrease of L* values and an increase of b* values when 1% beef plasma protein was added. Commercial surimi from Pacific whiting contains 1.2–1.5% beef plasma protein as enzyme inhibitor, to prevent gel-softening during a slow heating process (Park and Morrissey, 1994). Whiting surimi showed less green hue than pollock, indicating that the a* value was not affected by addition of beef plasma protein. Park (1994) also reported the independece of a* values of surimi gels upon protein additives. He further recommended the whiteness index, L* − 3b*. Reppond and Babbitt (1993) reported gel color values of Alaska

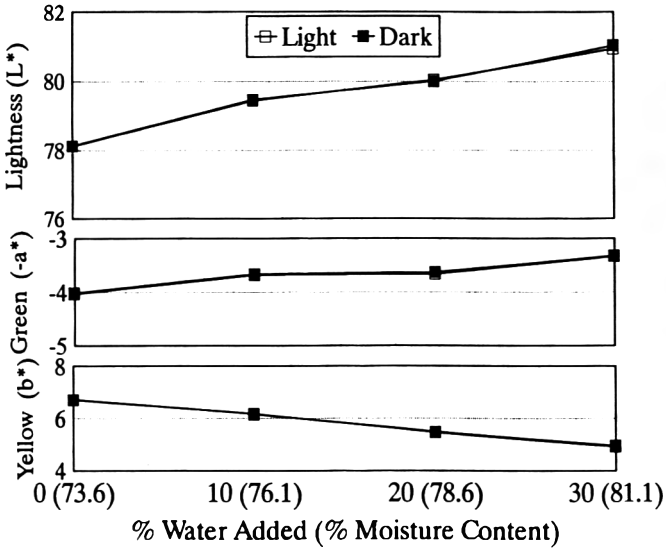


Fig. 6—Effects of room light on gel colors with levels of water addition.

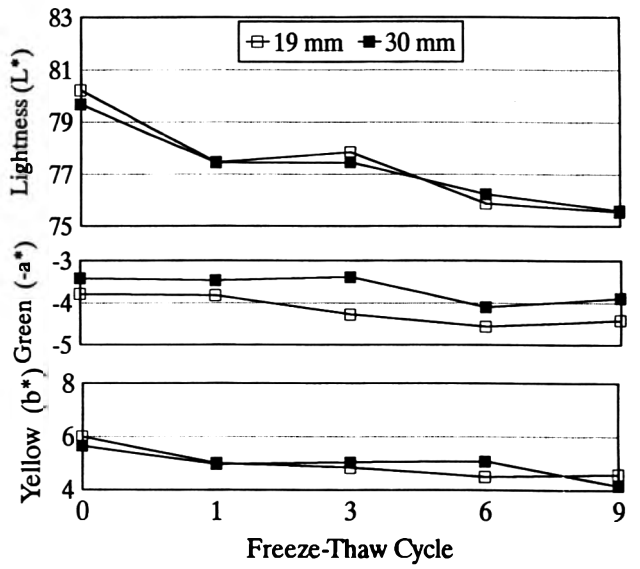


Fig. 8—Effects of sample size (diameter) on gel colors during freeze-thaw cycles.

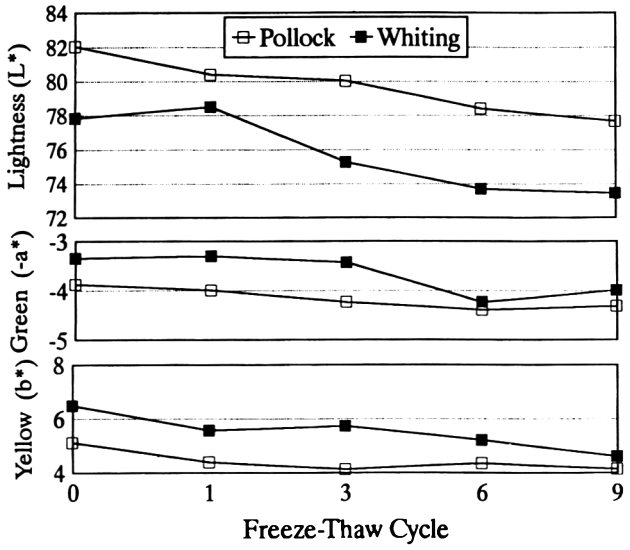


Fig. 7—Relation of species to gel colors during freeze-thaw cycles. Whiting surimi contained 1.2% beef plasma protein.

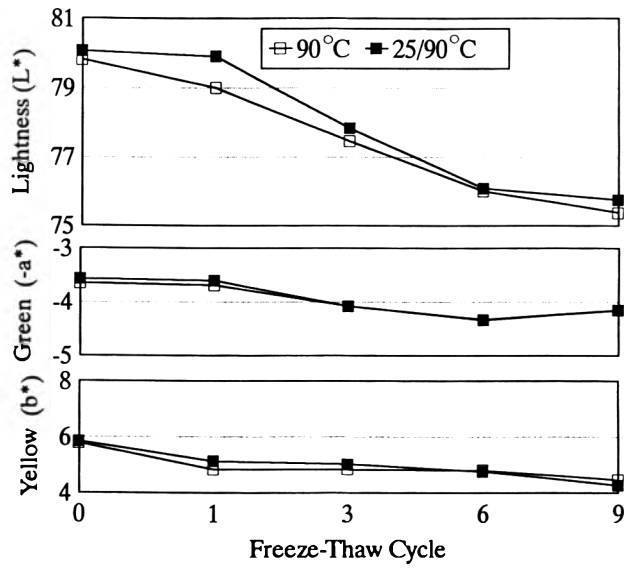


Fig. 9—Effects of heating temperatures on gel colors during freeze-thaw cycles. 90°C: heating at 90°C for 15 min. 25°/90°C: 3 hr setting at 25°C for 3 hr prior to heating at 90°C for 15 min.

pollock of 80.5 of L*, −2.1 of a*, and −2.5 of b*, while arrowtooth flounder surimi with 2% beef plasma protein were 77.6 of L*, −2.9 of a*, and 4.0 of b*.

Effects of physical conditions

Large-diameter gels looked lighter than small-diameter gels when no water had been added to the surimi (Fig. 3). However, with addition of water, small gels were lighter and more yellow than large gels ($P < 0.05$). Less green hue (−a*) was observed with large gels. Changes in a* values were very small regardless of water addition.

Heating temperature also affected L* and b* values (Fig. 4). Gels set at 25°C for 3 hr prior to heating at 90°C for 30 min were lighter (higher L*) when 0, 10, or 20% water was added. No difference occurred in green hue (−a*) between the two heating treatments. Yellow hue differences between heating treatments were also small.

The temperature of gels at the time of testing affected L* values ($P < 0.05$), while a* and b* values were not affected (Fig. 5). Gels at room temperature (25°C) were much lighter and had less green hue than cold gels (5°C). This suggested that gel temperature should be considered when the color of gels is measured. Light conditions of the room during measurement had no effect (Fig. 6). Whether the room was dark or well-lighted, measured colors of gels appeared to depend on the light source of the instrument.

Effects of freeze-thaw abuse

Freeze and thaw treatments affected colors of gels, making them darker. As freeze-thaw abuse continued, the color quality of gels was reduced ($P < 0.05$) (Fig. 7). Lightness (L*) decreased from ~32 to ~78 for pollock surimi gel and ~78 to ~74 for whiting surimi gel. Decreases of L* values during frozen storage were also observed with trout fillets (No and Storebakken, 1991), pollock mince (McFarland et al., 1992), and pike eel surimi (Hsu, 1990). Yellowness and greenness increased ($P < 0.05$) during freeze-thaw abuse, indicating that freezing darkened gels. These trends were very consistent, despite differences in sample size and heating temperature.

No significant difference in lightness and yellowness occurred between small (19 mm diameter) and large (30 mm diameter)

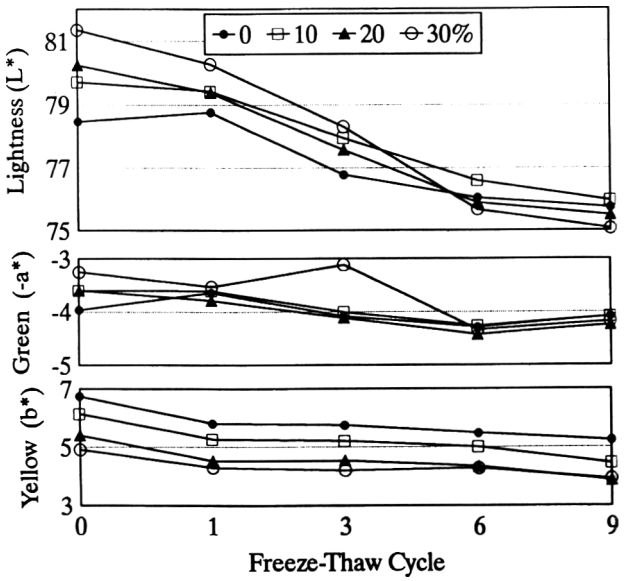


Fig. 10—Effects of levels of water addition on gel colors during freeze-thaw cycles.

samples during 9 cycles (Fig. 8). Gels with a two-stage heat treatment were slightly lighter than one-stage heated gels during 9 cycles (Fig. 8). Gels with a two-stage heat treatment were slightly lighter than one-stage heated gels during 9 cycles (Fig. 9). No difference ($P > 0.05$) occurred in greenness and yellowness between the two heating methods during 9 cycles. The various levels of water addition did not equally affect gel colors during freeze-thaw cycles (Fig. 10). However, overall lightness of gels decreased ($P < 0.05$) during nine cycles. The lightest gels before freeze-thaw were those with 30% water added during gel preparation, followed by 20%, 10% and 0% water.

After nine cycles, the effects of various levels of water addition on lightness were minimized. Gels with 30% water after nine cycles showed the lowest L* value. This was probably because gels with a higher moisture content were more susceptible to loss of water-holding capacity during freeze-thaw. Greenness increased slightly regardless of water addition during freeze-thaw. No difference occurred in green hue related to water additions. Yellow hue (b*) of the gels decreased slightly during nine cycles.

CONCLUSION

WATER ADDITION INCREASED LIGHTNESS (L*) and decreased yellowness (b*) of gels with all physical conditions treated. Pollock surimi was whiter than whiting surimi due to an enzyme inhibitor in the whiting sample. Smaller size showed higher L* and b*. Room temperature setting prior to 90°C slightly increased L* and b*. Gels measured at 25°C appeared lighter than those measured at 5°C. Room light condition at measurement did not affect L*, a*, and b*. Freeze-thaw abuse darkened colors of surimi gels. Gels had a significant reduction of L* after 9 cycles. The value of a* was less affected and independent of moisture levels and other physical conditions during freeze-thaw.

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Volatile Components in Salt-Fermented Fish and Shrimp Pastes

Y.J. CHA and K.R. CADWALLADER

ABSTRACT

Volatile compounds in salt-fermented anchovy, big-eyed herring, hair tail viscera, and shrimp pastes were compared by simultaneous steam distillation-solvent extraction/gas chromatography/mass spectrometry (SDE/GC/MS). A total of 155 volatile compounds were detected. Of these, 111 were positively identified consisting mainly of aldehydes, ketones, alcohols, esters, aromatics, nitrogen-, and sulfur-containing compounds. Lipid-derived components, such as aldehydes, alcohols, and esters, comprised the majority of volatile compounds from fish pastes, while heterocyclic nitrogen-containing compounds, such as pyrazines, were predominant in shrimp paste.

Key Words: fermented fish, shrimp paste, flavor volatiles, lipid-derived components, heterocyclic compounds

INTRODUCTION

SALT-FERMENTED FISH PRODUCTS, such as paste and sauce, are manufactured in vast quantities for human consumption throughout Asia. Fish pastes are consumed especially as a condiment for rice dishes. Traditionally, more than 30 kinds of fermented fish pastes have been consumed as seasonings added to Kimchi, a typical Korean pickled vegetable, and also as an important protein and fat source in Asian diets. Most fish pastes contain 25–30% salt and are fermented for over 6 mo. The characteristic aroma and taste are primarily due to protein and lipid degradation by autolytic and bacterial enzymes during fermentation (Beddows et al., 1980; Saisithi et al., 1966). Studies on the taste of fish pastes have been reported. Cha and Lee (1985) examined taste-active compounds of fermented anchovy and yellow corvenia pastes and concluded that free amino acids, nucleotides, and related compounds were most important. Similarly, Chung and Lee (1976) and Lee et al. (1981) reported that those components were primarily responsible for the taste of fermented shrimp and sardine pastes, respectively. Several microbiological studies on fermented fish pastes also have been reported (Saisithi et al., 1966; Lee, 1969; Mori et al., 1979).

As a consequence of the modern evolution of diets, Asian seafood industries have shifted focus from traditional salt-fermented fish products toward more convenient and healthier seasoning agents. This trend has in turn initiated studies of fermented fish products to better understand and improve their composition and potential impact on human health. Several studies attempted to produce low salt-fermented fish products (Cha et al., 1983; Chayovan et al., 1983). However, the volatile flavor of fermented fish products has not been fully investigated, except for a few reports on fish and shrimp sauces (Nonaka et al., 1975; Choi et al., 1983; Choi and Kato, 1984). A thorough understanding of fish paste volatile flavor is essential if alternative products are to be successful. Our objective was to identify and compare volatile components of several types of fermented fish pastes.

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MATERIALS & METHODS

Materials

Four kinds of popular salt-fermented fish pastes, anchovy (*Engraulis japonica*), big-eyed herring (*Harengula zunasi*), shrimp (*Acetes chinensis*), and hair tail (*Trichiurus japonica*) viscera, were obtained from a fish market in Masan, Korea. Samples were homogenized using a Waring Blendor (Waring Products Co., Winsted, CT) before analysis. Standard flavor compounds were obtained from commercial sources.

Composition analysis

Moisture content was determined by an oven drying method (AOAC, 1980). Crude protein was determined by a micro-Kjeldahl method (AOAC, 1980). Lipid and ash were determined by Soxhlet extraction (AOAC, 1980) and dry ashing (AOAC, 1980), respectively. Salt content was measured by the Mohr method (AOAC, 1980), and pH was measured using a pH meter (model No. 630, Fisher Scientific, Pittsburgh, PA).

Simultaneous steam distillation-solvent extraction (SDE)

Homogenized fish paste (500g) plus 90.8 µg of internal standard (2,4,6-trimethylpyridine, TMP) and 1.5 L of distilled water were extracted with 100 mL of redistilled diethyl ether for 3 hr using a Likens and Nickerson (1964) type SDE apparatus (Cat. No. K-523010-0000, Kontes, Vineland, NJ). Further details were described by Tanchotikul and Hsieh (1991). Extracts were concentrated to 3 mL under a gentle stream of nitrogen. Triplicate extractions were carried out for each sample.

Gas chromatography/mass spectrometry (GC/MS)

GC/MS system consisted of a Hewlett-Packard 5790 GC/HP 5970B mass selective detector (MSD) (Hewlett-Packard Co., Palo Alto, CA). Three µL of each SDE extract were injected in the splitless mode (155°C injector; 30 sec valve delay) into a fused silica open-tubular (FSOT) column (Supelcowax 10, 60m length × 0.25 mm i.d. × 0.25 µm film thickness; Supelco, Inc., Bellefonte, PA). Oven temperature was programmed from 40°C to 175°C at 2°C/min with initial and final hold times of 5 and 30 min, respectively; oven temperature was then further increased to 195°C at 5°C/min and maintained 25 min. Electron ionization voltage was 70 eV. Mass range was 33-300 a.m.u., electron multiplier voltage was 2000 V, and scan rate was 1.6 sec^{-1}. Other details of the GC/MSD procedure were described by Cha et al. (1993). Duplicate analyses were performed on each SDE extract.

Identification and quantitation of compounds

Positive identifications were based on comparison of retention indices (RI) (van den Dool and Kratz, 1963) and mass spectra of unknowns with authentic compounds. Tentative identifications were based on standard MS library data (Hewlett-Packard Co., 1988).

Positively identified compounds were quantified using calibration curves of amount ratios (compound/I.S.) vs peak area ratios (compound/I.S.) under identical chromatographic conditions. The relative abundance of a tentatively identified compound was based on the ratio of its total ion peak area to that of the I.S.

RESULTS & DISCUSSION

THE PROXIMATE COMPOSITION of salt-fermented fish and shrimp pastes were compared (Table 1). Anchovy and big-eyed herring pastes were higher in lipid content, while hair tail viscera and

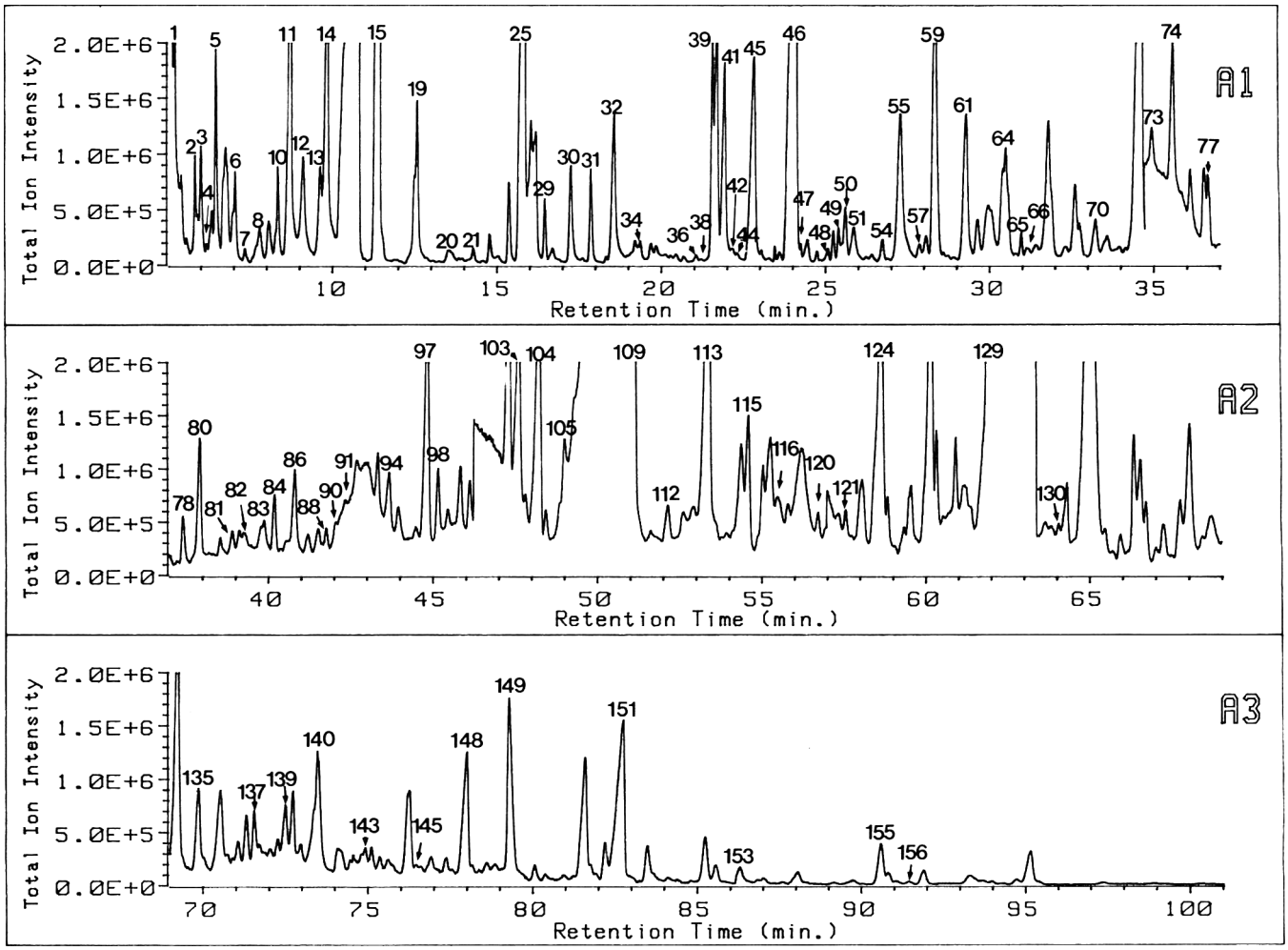


Fig. 1—Total ion chromatogram of volatile components in salt-fermented anchovy paste (A1-A3). Peak numbers correspond to those listed in Table 2.

Table 1—Proximate composition of Korean salt-fermented fish and shrimp pastes

	% Concentration (w/w)					
	Moisture	Protein	Lipid	Ash	Salinity	pH
Anchovy	53.3 ± 0.9[a]	12.3 ± 0.5	11.1 ± 2.7	17.4 ± 0.9	11.3 ± 0.7	6.68
Big eyed herring	60.9 ± 1.3	9.8 ± 0.5	10.8 ± 0.6	17.9 ± 0.6	13.2 ± 0.7	7.09
Hair tail viscera	56.6 ± 1.6	12.3 ± 0.9	6.2 ± 0.5	20.3 ± 1.8	19.4 ± 1.1	6.23
Shrimp	60.2 ± 0.3	7.0 ± 0.2	2.1 ± 0.5	28.2 ± 1.0	22.1 ± 0.8	8.50

[a] Mean value ± standard deviation (n = 3).

shrimp paste were higher in salinity. Moisture contents were ≈ 53%-60% for all samples. Total ion chromatograms (TICs) of volatile components in anchovy, big eyed herring, hair tail viscera pastes were similar. Typical TICs of anchovy and shrimp paste volatiles were compared (Fig. 1 and 2). A total of 155 volatile compounds were detected of which 111 were positively identified (Table 2). Eighty-five compounds were detected in anchovy paste and in big eyed herring paste, 82 in hair tail viscera paste, and 72 in shrimp paste.

As shown (Table 2) the number and abundance of aldehydes in all fish pastes was high; however, in contrast, number and abundance of aldehydes in shrimp paste were low. Aldehydes, such as benzaldehyde, 2- and 3-methylbutanal and (E,E)-2,4-heptadienal were high in all fish pastes. The compounds 2- and 3-methylbutanal were the most abundant aldehydes in shrimp paste. Benzaldehyde, with a pleasant almond, nutty, and fruity aroma (Vejaphan et al., 1988), was found in all pastes. The compounds (E,E)-2,4-heptadienal and hexanal were notably high in big eyed herring paste. Certain aldehydes, such as (E)-2-hexenal and (Z)-4-heptenal were detected only in hair tail viscera paste. The alkanals, alkenals, and alkadienals may have originated from lipid oxidation during fermentation (Karahadian and Lindsay, 1989). Most fish oils contain a relatively high proportion of w-3 fatty acids (18:3, 20:5, 20:6) (Love, 1992). Cha (1985) reported that anchovy paste contained high amounts of w-3 and w-6 fatty acids, which are highly susceptible to lipid oxidation. Most of the alkanals and enals are known to contribute fatty-oily, slightly rancid odors, while the dienals contribute pleasant fried-fatty aromas (Vejaphan et al., 1988; Heath and Reineccius, 1986). Ho et al. (1989), however, reported that some aldehydes with unpleasant odors, may act as important precursors of heterocyclic compounds.

2,3-Butanedione, 2-heptanone, 3-hydroxy-2-butanone, 2-nonanone, and (E,E)-3,5-octadien-2-one (tentatively identified) were the most abundant ketones found in all fish pastes. Levels of (E)-3-penten-2-one and 2-undecanone were notably high in big-eyed herring paste, while that of β-ionone was high in both big-eyed herring and hair tailed visceral pastes. 2,3-Butanedione, found in all pastes, was reported to contribute an intense buttery and desirable aroma in crustacea (Tanchotikul and Hsieh, 1989) and was previously found to be in high amounts in shrimp paste (Choi and Kato, 1984). (E,E)-3,5-Octadien-2-

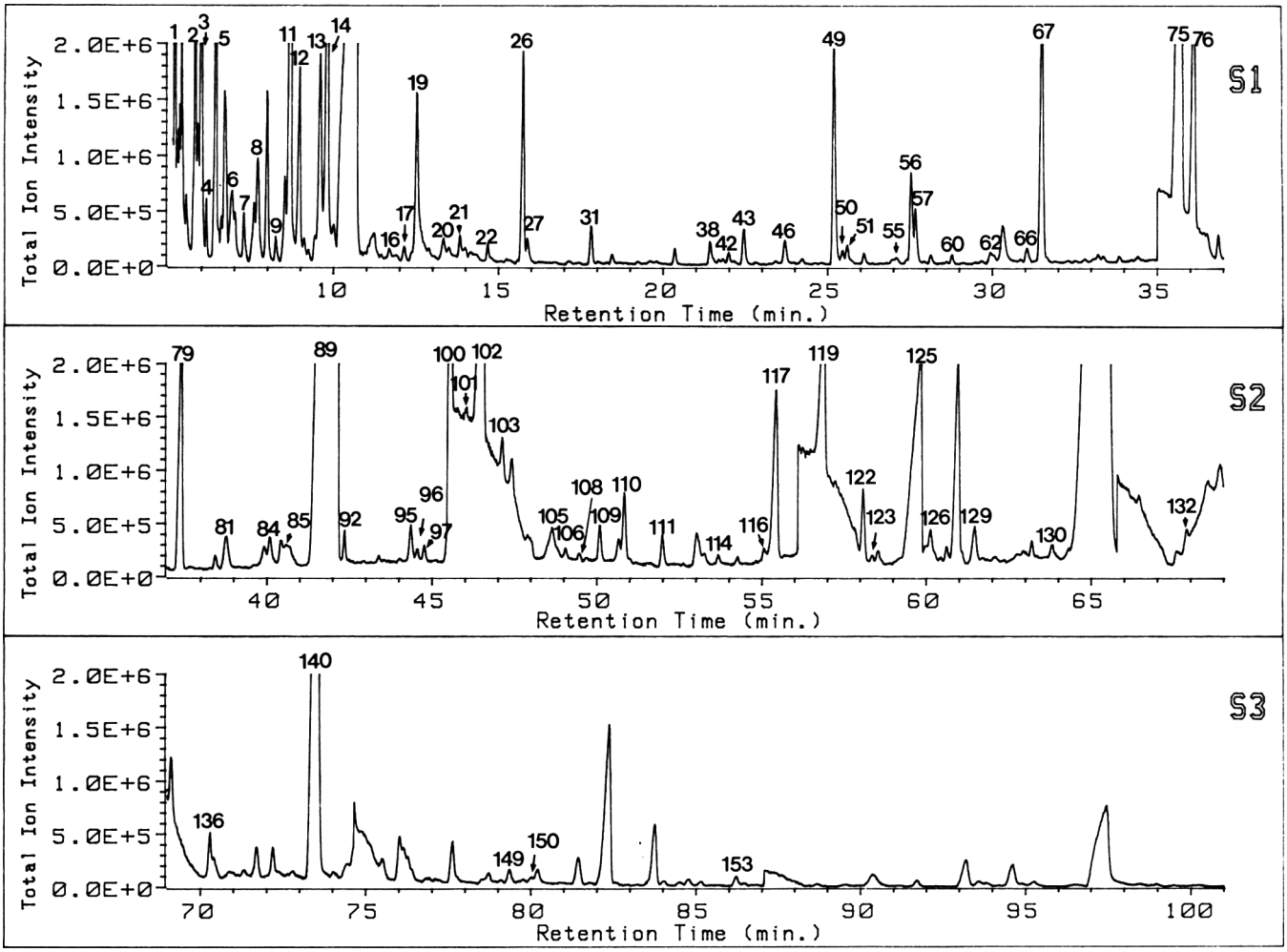


Fig. 2—Total ion chromatogram of volatile components in salt-fermented shrimp paste (S1-S3). Peak numbers correspond to those listed in Table 2.

one, contributing a fatty-fruity odor, may be important in fermented fish pastes. This compound has been reported as a lipid oxidation product in fish (Karahadian and Lindsay, 1989) and in roasted shrimp (Kubota et al., 1986). Geranylacetone, a terpene derivative, has been shown to occur in plants (Chen and Ho, 1989). This compound was detected only in shrimp paste and may have originated from the diet, e.g., plankton.

The alcohols 1-penten-3-ol, 3-methyl-1-butanol, 1-octen-3-ol, and (E)-2-penten-1-ol were in high abundance in all pastes, except shrimp. Alcohols were low in number and amounts in shrimp paste, with the exception of 2-ethyl-1-hexanol. This corresponds to the low overall number and abundance of aldehydes detected in shrimp paste compared with other fish pastes and was hypothesized to be due to the lower lipid content of shrimp paste. Among fish pastes, a higher amount of alcohols was found in big-eyed herring paste. Despite their high amounts, alcohols might not have an impact on fish paste flavor because of their high flavor thresholds (Heath and Reineccius, 1986).

Among the aromatic/phenolic compounds identified, toluene was in highest abundance in shrimp paste, while phenol was highest in fish pastes. Carotenoids are hypothesized to be the precursors of toluene and benzene derivatives found in fish and shellfish pastes (Borenstein and Bunnell, 1969). Some undesirable aromatic hydrocarbons, such as toluene and phenol, have been reported in other seafoods (Vejaphan et al., 1988; Cha et al., 1993).

The majority of nitrogen-containing compounds was found in shrimp paste, while only a few were found in fish pastes. Abundance of nitrogen-containing compounds was > 50% of the total ion peak area of volatiles found in shrimp paste (data not shown) and comprised > 25% of the identified compounds. Eleven pyrazines were positively identified. Trimethylpyrazine was in highest abundance in shrimp paste. Only one pyrazine, 2-ethyl-3,6-dimethylpyrazine, was found in big-eyed herring and hair tail viscera pastes. No pyrazines were detected in anchovy paste. Pyrazines have been reported to contribute nutty, roasted, and toasted aromas to many foods (Maga and Sizer, 1973). Pyrazines were reported to be formed by Maillard reaction through Strecker degradations from various nitrogen sources such as amino acids (Shibamoto et al., 1979; Wong and Bernhard, 1988; Koehler and Odell, 1970). We did not expect that more pyrazines would be detected in shrimp paste, since the fish pastes have been shown to have greater amounts of free amino acids (Cha and Lee, 1985); however, the higher pH of shrimp paste (Table 1) would favor pyrazine formation (Koehler and Odell, 1970). Alkylpyridines, having unpleasant aromas, were found in all pastes in relatively low amounts.

A large number of esters were found in fermented fish pastes, many of which were tentatively identified. A series of butanoate esters was in high amounts, especially in big-eyed herring paste. Esters may have arisen from esterification of various alcohols with carboxylic acids formed via microbial and enzymatic decomposition of lipids. Esters have been found in most fermented seafoods (Sanceda et al., 1990; Josephson et al., 1987). Ethyl butanoate, which was in high amounts in both anchovy and big-eyed herring pastes, provides a characteristic flavor to cheese products (Lee et al., 1986). Additional research is needed to determine the effects of esters in the flavor of fermented fish pastes.

Dimethyldisulfide and dimethyltrisulfide as well as the tentatively identified compounds isobutylisopropylsulfide and 3,5-dimethyl-1,2,4-trithiolane were most abundant among the

Table 2—Volatile components in Korean salt-fermented fish and shrimp pastes

Peak no.	Compound name by class	RI[b]	Mean concentration (ng/g)[a]			
			Anchovy	Big eyed herring	Hair tail viscera	Shrimp
	Aldehydes (25)					
10	Butanal	874	2230 (±49%)	6420 (±40%)	1150 (±64%)	
13	2-Methylbutanal	917	2650 (±52%)	43800 (±59%)	4910 (±58%)	5870 (±66%)
14	3-Methylbutanal	922	9720 (±54%)	77100 (±41%)	22100 (±99%)	12400 (±64%)
27	(E)-2-Butenal	1042			837 (±124%)	201 (±50%)
32	Hexanal	1081	951 (±54%)	5740 (±40%)	1080 (±54%)	
34	2-Methyl-(E)-2-butenal	1094	214 (±68%)			
41	(E)-2-Pentenal	1130	1170 (±45%)		972 (±55%)	
52	Heptanal	1184		1820 (±38%)	843 (±64%)	
58	(E)-2-Hexenal	1215			688 (±57%)	
62	(Z)-4-Heptenal	1239			477 (±48%)	143 (±41%)
71	Octanal	1289		999 (±29%)	400 (±67%)	
77	(E)-2-Heptenal	1328	760 (±63%)		1400 (±165%)	
87	Nonanal	1393		707 (±46%)	693 (±76%)	
88	(E,E)-2,4-Hexadienal	1398	491 (±82%)	1570 (±83%)		
94	(E)-2-Octenal	1426	262 (±38%)	566 (±50%)	226 (±60%)	
104	(E,E)-2,4-Heptadienal	1494	1080 (±38%)	5230 (±42%)	1040 (±60%)	
109	Benzaldehyde	1522	6910 (±62%)	2560 (±59%)	17800 (±59%)	222 (±17%)
115	(E,Z)-2,6-Nonadienal	1589	377 (±40%)	2120 (±51%)	486 (±50%)	
121	Phenylacetaldehyde	1634	952 (±43%)			
128	(E,E)-2,4-Nonadienal	1702		320 (±42%)		
131	2-Undecenal*	1749			1120 (±99%)	
133	(E,E)-2,4-Decadienal	1807		1610 (±37%)	619 (±33%)	
142	Tetradecanal*	1918			2250 (±116%)	
143	α-Ethylidene-phenylacetaldehyde*	1928	176 (±40%)		145 (±46%)	
154	Hexadecanal*	2126			1820 (±171%)	
	Ketones (16)					
19	2,3-Butanedione	981	512 (±57%)	260 (±46%)	2470 (±75%)	791 (±67%)
21	4-Methyl-2-pentanone	1012	43.6 (±32%)			93.6 (±45%)
23	1-Penten-3-one	1023		1080 (±88%)	389 (±56%)	
30	2,3-Pentanedione	1062	669 (±45%)	1450 (±47%)		
40	(E)-3-Penten-2-one	1128		1910 (±44%)	119 (±84%)	
50	2-Heptanone	1181	146 (±20%)	455 (±31%)	115 (±60%)	42.0 (±33%)
65	3-Octanone	1252	74.5 (±46%)	152 (±32%)		
69	2-Octanone	1280			25.7 (±42%)	
70	3-Hydroxy-2-butanone	1282	355 (±34%)	714 (±48%)	193 (±49%	
78	3-Hydroxy-3,5-dimethyl-2-hexanone*	1338	200 (±29%)			
86	2-Nonanone	1386	323 (±34%)	515 (±25%)	247 (±47%	
113	(E,E)-3,5-Octadiene-2-one*	1568	2200 (±43%)	2770 (±48%)	427 (±44%	
116	2-Undecanone	1598	281 (±54%)	887 (±48%)		49.1 (±33%)
123	Acetophenone	1646				64.8 (±24%)
136	Geranylacetone	1848				250 (±109%)
144	β-Ionone	1935		463 (±29%)	923 (±56%	
	Alcohols (22)					
24	(E)-2-Butanol	1029		3490 (±49%)	1060 (±70%	
35	3-Pentanol	1108		670 (±35%)	1080 (±53%	
37	2-Pentanol	1119		725 (±60%)		
45	Butanol	1143	2420 (±38%)	4050 (±56%)		
46	1-Penten-3-ol	1160	6330 (±42%)	6140 (±40%)	3130 (±50%)	223 (±45%)
55	3-Methyl-1-butanol	1204	1100 (±39%)	10800 (±36%)	1170 (±59%)	39.7 (±41%)
63	Pentanol	1243		1890 (±41%)	346 (±48%	
64	1-Hexen-3-ol	1246	603 (43%)			
72	Cyclopentanol	1304			437 (±52%	
73	(Z)-2-Penten-1-ol	1305	2030 (±59%)	3690 (±55%)		
74	(E)-2-Penten-1-ol	1314	4420 (±35%)	4240 (±33%)	2165 (±35%)	
80	Hexanol	1346	710 (±26%)	2640 (±35%)	483 (±53%)	
91	(E)-2-Hexen-1-ol	1406	805 (±53%)			
97	1-Octen-3-ol	1444	1540 (±34%)	4780 (±40%)	968 (±47%)	135 (±34%)
98	Heptanol	1448	527 (±51%)	1970 (±62%)	392 (±44%)	
103	2-Ethyl-1-hexanol	1485	1560 (±35%)	630 (±50%)	390 (±37%)	1840 (±42%)
112	Octanol	1552	170 (±68%)	644 (±48%)	213 (±96%)	
124	2-Furanmethanol	1652	3260 (±28%)		10900 (±76%)	
137	Benzenemethanol	1870	389 (±38%)	1230 (±60%)	352 (±41%)	
141	2-Phenylethanol	1906		8080 (±27%)	2430 (±60%)	
146	Dodecanol	1959		185 (±82%)		
155	1,4-Tetradecanediol*	2131	221 (±61%)			
	Aromatic compounds (13)					
26	Toluene	1039			547 (±81%)	777 (±52%)
38	Ethylbenzene	1121	76.6 (±189%)		38.3 (±86%)	72.5 (±32%)
42	p-Xylene	1134	79.4 (±142%)			39.0 (±39%)
43	m-Xylene	1136		378 (±45%)	124 (±92%)	99.9 (±42%)
51	o-Xylene	1183	132 (±48%)	102 (±72%)		58.6 (±61%)
66	Styrene	1254	69.0 (±16%)	223 (±53%)		180 (±159%)
96	1,4-Dichlorobenzene	1438				124 (±29%)
130	Naphthalene	1738	261 (±43%)	187 (±91%)	693 (±62%)	201 (±32%)
134	4-(Methylthio)phenol*	1813			291 (±90%)	
149	Phenol	1997	1540 (±28%)	39,100 (±38%)	2400 (±56%)	256 (±82%)
150	Phenylether*	2008				109 (±65%)
152	o-Cresol	2072		18.6 (±73%)		
153	3-tert-Butyl-4-hydroxyanisole*	2087	67.2 (±76%)	390 (±37%)		56.3 (±16%)
	N-Containing compounds (22)					
49	Pyridine	1178	100 (±40%)	179 (±32)	67.9 (±74%)	758 (±43%)
56	Pyrazine	1206				630 (±36%)
57	2-Methylpyridine	1211	146 (±40%)	203 (±83%)		292 (±58%)

Table 2—(Continued)

Peak no.	Compound name by class	RI[b]	Mean concentration (ng/g)[a]			
			Anchovy	Big eyed herring	Hair tail viscera	Shrimp
60	4,5-Dihydro-4,5-dimethyl-1H-pyrazole*	1222				30.9 (±24%)
67	2-Methylpyrazine	1259				1300 (±38%)
75	2,5-Dimethylpyrazine	1315				4180 (±18%)
76	2,6-Dimethylpyrazine	1320				2190 (±43%)
79	2,3-Dimethylpyrazine	1338				1410 (±33%)
81	2,4,6-trimethylpyridine (I.S.)[c]	1359				
83	3-Ethylpyridine	1373	319 (±43%)	379 (±50%)	133 (±117%)	
85	2-Ethyl-5-methylpyrazine	1382				207 (±13%)
89	Trimethylpyrazine	1401				17900 (±31%)
92	5-Ethyl-2-methylpyridine*	1406				140 (±43%)
95	2-Ethyl-3,6-dimethylpyrazine	1436		843 (±33%)	212 (±36%)	210 (±17%)
100	2-Ethyl-3,5-dimethylpyrazine	1453				1410 (±59%)
102	Tetramethylpyrazine	1467				5360 (±30%)
106	3,5-Diethyl-2-methylpyrazine	1504				92.6 (±49%)
108	1H-Pyrrole	1510				79.6 (±37%)
110	2-Allyl-3-methylpyrazine*	1531				325 (±10%)
111	2-Methyl-1H-pyrrole*	1548				154 (±16%)
114	1-Methyl-2(1H)-pyridinone*	1574				61.7 (±68%)
139	3-Acetoxypyridine*	1887	343 (±60)		236 (±44%)	
140	Hexamethylenetetramine*	1904	1540 (±68%)		3820 (±102%)	3990 (±24%)
	Esters (21)					
11	Ethyl acetate	893	3490 (±38%)	1660 (±34%)	2340 (±74%)	3750 (±33%)
18	Isopropyl propanoate	971		604 (±37%)		
25	Ethyl butanoate	1039	3740 (±54%)	10400 (±59%)	74.9 (±46%)	
28	Isopropyl butanoate	1043		6920 (±46%)		
29	S-Methyl ethanethioate*	1049	441 (±37%)			
36	2-Methylpropyl propanoate*	1119	30.9 (±47%)			
39	Propyl butanoate	1125	993 (±52%)	2020 (±62%)		
44	Ethyl pentanoate	1137	67.3 (±136%)			
47	2-Methylpropyl butanoate*	1163	54.5 (±90%)			
53	Pentyl propanoate*	1190		933 (±54%)		
54	S-Methyl butanethioate*	1195	94.4 (±27%)	3830 (±39%)		
59	Butyl butanoate	1218	1230 (±32%)	4240 (±38%)		
68	Pentyl butanoate*	1270		7770 (±74%)		
93	Hexyl butanoate	1418		212 (±49%)		
132	Geranyl acetate*	1805				281 (±13%)
135	Ethyl dodecanoate*	1840	670 (±68%)	1650 (±23%)	516 (±65%)	
138	Ethyl-3-phenyl propionate*	1882			875 (±61%)	
147	2-Phenylethyl butanoate*	1963		1680 (±37%)		
148	Ethenylcyclopentane acetate*	1978	665 (±43%)			
151	Ethyltetradecanoate	2042	2080 (±100%)	1370 (±27%)	1340 (±100%)	
156	Ethylhexadecanoate*	2139	29.0 (±56%)			
	S-Containing compounds (12)					
31	Dimethyldisulfide	1071	540 (±38%)	32900 (±32%)	182 (±73%)	224 (±47%)
33	2-Methylthiophene	1089		321 (±35%)		
48	2-Ethylthiophene*	1174	67.2 (±43%)			
82	2-Propylthiophene*	1364	241 (±39%)	343 (±42%)		
84	Dimethyltrisulfide	1377	390 (±30%)	3790 (±45%)		312 (±61%)
99	3-(Methylthio)-propanal*	1452			399 (±51%)	
107	Isobutylisopropylsulfide*	1506		4970 (±31%)		
117	3,5-Dimethyl-1,2,4-trithiolane(isomer)*	1601				792 (±26%)
119	3,5-Dimethyl-1,2,4-trithiolane(isomer)*	1622		2590 (±29%)	1550 (±91%)	2660 (±81%)
122	2-Acetylthiazole	1642				802 (±36%)
126	3-Thiophenecarboxaldehyde	1674				227 (±26%)
145	Benzothiazole	1953	124 (±100%)		247 (±38%)	
	Furans (3)					
15	2-Ethylfuran	957	5160 (±58%)	15700 (±80%)	4440 (±75%)	
61	2-Pentylfuran	1231	664 (±31%)	3040 (±43%)	682 (±50%)	
101	2-Furancarboxaldehyde	1460		3760 (±49%)		599 (±86%)
	Miscellaneous compounds (21)					
1	Heptane	700	662 (±111%)	1790 (±141%)	948 (±36%)	1560 (±69%)
2	2-Methylheptane*	753	358 (±43%)	1670 (±33%)	1090 (±92%)	1040 (±60%)
3	1,1,2,3-Tetramethylcyclopropane*	767	510 (±52%)	1550 (±33%)	1730 (±114%)	1460 (±63%)
4	Ethylcyclopropane*	780	49.0 (±70%)			183 (±61%)
5	Octane	803	531 (±43%)	1740 (±38%)	1640 (±69%)	1230 (±58%)
6	1-Ethyl-2-methylcyclopentane*	825	726 (±139%)		474 (±57%)	296 (±23%)
7	1,2-Dimethyl-(E)-cyclohexane*	837	61.7 (±44%)	123 (±41%)	123 (±54%)	187 (±45%)
8	2-Methyloctane*	855	249 (±28%)	806 (±36%)	543 (±76%)	492 (±46%)
9	Propylcyclopentane*	872				90.8 (±34%)
12	Nonane	903	332 (±40%)	1540 (±30%)	999 (±67%)	529 (±47%)
16	3-Methylnonane*	964		392 (±30%)	65.4 (±97%)	96.2 (±34%)
17	Propylcyclohexane*	972				78.1 (±30%)
20	Decane	1002	46.8 (±33%)	183 (±43%)	45.3 (±28%)	57.8 (±53%)
22	Chloroform	1020				133 (±18%)
90	Tetradecane	1402	303 (±47%)		655 (±64%)	
105	Pentadecane	1502	1204 (±65%)			390 (±30%)
118	Hexadecane	1605			1850 (±66%)	
120	Butanoic acid	1622	243 (±49%)			
125	2,6,10,14-Tetramethylpentadecane	1674			8710 (±138%)	7160 (±37%)
127	2,6,10,14-Tetramethylhexadecane*	1690		18300 (±29%)	7610 (±78%)	
129	Heptadecane	1703	2080 (±64%)		2520 (±113%)	192 (±52%)

[a] Mean concentration from three SDE extractions and two injections of each extract. Numbers in parentheses represent relative standard deviations.

[b] Retention index.

[c] Internal standard.

* Compound tentatively identified by mass spectrum.

sulfur-containing compounds. Amount of dimethyldisulfide, with a strong and sulfurous odor, was highest in big-eyed herring paste. Presence of these S-containing compounds may affect the overall flavor because of their low thresholds (Buttery et al., 1976), e.g., 3,5-dimethyl-1,2,4-trithiolane isomers in shrimp paste. These compounds contributed an onion-like odor in boiled Antarctic krill (Kubota et al., 1980) and can be produced by reaction of aldehydes with hydrogen sulfide (Ho et al., 1989). Two thiazoles were identified; 2-acetylthiazole in shrimp paste, and benzothiazole in anchovy and hair tailed viscera paste. 2-Acetylthiazole was reported to have a nutty and popcorn-like aroma (Ho and Carlin, 1989).

Furans have been reported to have burnt, sweet, bitter, and coconut-like flavor in some foods (Maga, 1979). Although the number of these thermally generated compounds was small, they were found in high amounts. Alkanes were the major volatile compounds found among miscellaneous compounds. We hypothesized that these compounds do not contribute to the characteristic flavor of fermented fish paste because of their high thresholds.

In summary, aldehydes, ketones, alcohols, and esters, products of lipid oxidation and enzyme-mediated lipid and protein breakdown during fermentation, were in high abundance in fish pastes, regardless of fish species. Esters were in high amounts in anchovy and big-eyed herring paste, while aldehydes were predominant in hair tail viscera paste. Aldehydes, ketones, and esters were believed to contribute characteristic aromas to the overall flavor of salt-fermented anchovy, big-eyed herring and hair tail viscera pastes. Abundance of the lipid-derived compounds was comparatively lower in shrimp pastes. Nitrogen-containing compounds comprised the major fraction of volatile compounds identified in fermented shrimp paste. They are probably important in the characteristic flavor of shrimp paste.

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Sodium Acetate and Bifidobacteria Increase Shelf-Life of Refrigerated Catfish Fillets

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ABSTRACT

Microbiological and sensory evaluations of catfish fillets treated with sodium acetate (SA) and bifidobacteria were studied at 4°C. Cultures of *Bifidobacterium adolescentis, B. infantis,* or *B. longum* (2.5% V/W) extended lag phase, but not generation time of aerobic bacteria. One-half percent (W/W) SA increased lag phase and generation time. SA and bifidobacteria reduced counts more than either alone, extending shelf-life by 3 days. Amount of culture (1.5-4.5%) or culture age (24-72 hr) had no effect on inhibition. Treated fillets odor and appearance resembled fresh fillets for up to 6 days, while untreated fillets were unacceptable after 3 days. SA, either alone or combined with bifidobacteria, extended refrigerated shelf-life of catfish fillets.

Key Words: sodium acetate, bifidobacteria, catfish, shelf-life

INTRODUCTION

QUALITY AND SAFETY of refrigerated foods have been enhanced by preventing growth or destroying aerobic spoilage bacteria and foodborne pathogens during storage and handling using food additives and biopreservatives (Gilliland and Ewell, 1983; Lindgren and Dobrogosz, 1989; Kim and Hearnsberger, 1994). Biopreservatives such as lactic acid bacteria (LAB) or their metabolites in refrigerated foods may suppress aerobic bacteria that cause food spoilage (Raccach and Baker, 1978; Gilliland and Ewell, 1983; Schaack and Marth, 1988; Lindgren and Dobrogosz, 1989; Laroia and Martin, 1990; Modler et al., 1990; Hughes and Hoover, 1991; Ray, 1992; Kim and Hearnsberger, 1994).

Lindgren and Dobrogosz (1989) reported that a combination of *Lactobacillus leuteri* and 250 mM glycerol inhibited aerobic spoilage bacteria in herring fillets stored in N_2 at 5°C for 6 days. Raccach et al. (1979) and Schaack and Marth (1988) showed similar effects with LAB on food spoilage bacteria and pathogens in poultry meat and skim milk, respectively. Raccach et al. (1979) found that use of *Pediococcus cerevisiae* (Accel^R) and *Lactobacillus plantarum* (Lactacel^R) in refrigerated deboned poultry meat decreased the microbial population, reduced off-odor, and extended shelf-life by 2 days. Kim and Hearnsberger (1994) reported that combination of *Lactococcus lactis* ssp. *cremoris* ATCC 19257 with sodium acetate and potassium sorbate effectively inhibited growth of gram-negative bacteria in refrigerated catfish fillets.

Bifidobacteria may control food spoilage bacteria and foodborne pathogens through production of lactic and acetic acids as well as other antibiotic substances (Laroia and Martin, 1990; Modler et al., 1990; Hughes and Hoover, 1991; Ray, 1992). Previous work in our laboratories has shown that sodium acetate was effective in suppressing growth of aerobic spoilage bacteria on refrigerated catfish fillets (Kim and Hearnsberger, 1994). Because no studies have been reported on the combined effects of preservatives and bifidobacteria as an antimicrobial treatment of fish or fish products, our objective was to evaluate shelf-life of refrigerated (4°C) channel catfish fillets surface-treated with sodium acetate and selected bifidobacteria.

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MATERIALS & METHODS

Preparation of bifidobacteria cultures

Bifidobacterium infantis ATCC 15697 and *B. longum* ATCC 15707 were obtained from the American Type Culture Collection (Rockville, MD). *B. adolescentis* 9H Martin was obtained from the culture collection of Dr. J. H. Martin, Mississippi State University. Stock cultures were maintained in sterile skim milk medium (SM; 10% skim milk, 0.5% yeast extract, and 0.5% glucose) at 37°C for 24 hr in an anaerobic jar (Gas-Pak; BBL). For fish inoculation, cultures were prepared by 3 successive 1:10 transfers at 24-hr intervals into fresh SM incubated as above. Bifidobacteria cultures in SM (6.0×10^7 to 1.0×10^8 CFU/mL) were added at a given percentage (V/W) to fresh catfish fillets. Final SM pH was 4.4–4.8.

Catfish preparation and treatment

Fresh channel catfish (*Ictalurus punctatus*) fillets were obtained from a commercial source, transported to MSU on ice, and used within 3 hr. For each treatment, 2 kg of fillets (average weight 110 g/fillet) were placed in a precleaned (Jettson General Purpose Cleaner, Diversy Group, Cincinnati, OH) and sanitized (hot water at 88°C) tumbler (Polymaid Model SS 350, Largo, FL) at room temperature (≈23°C). Bifidobacteria cultures and/or sodium acetate (SA; Fisher Scientific Co., Norcross, GA) were added, individually or combined, to the tumbler and fillets were tumbled for 15 min at 18 rpm. Untreated controls were tumbled without preservatives. The tumbler was cleaned and sanitized between treatments. Duplicate experimental trials consisted of the following: (a) 0 or 0.5% SA (W/W), (b) 0 or 2.5% *B. adolescentis, B. infantis,* or *B. longum* (V/W), (c) 0 or 2.5% (V/W) of *B. adolescentis, B. infantis,* or *B. longum* combined with 0 or 0.5% SA, (d) 0 or 0.5% SA combined with 1.5%, 2.5%, 3.5% or 4.5% *B. infantis*, (e) 0 or 0.5% SA combined with 2.5% *B. infantis* incubated for 24, 48, or 72 hr before addition. Treated catfish fillets were removed from the tumbler and stored individually in "Ziploc^R" (DowBrands L.P., Indianapolis, IN) bags aerobically at 4°C. Storage bag gas transmission rate was 1.65 cc/cm^2 per mil in 24 h at 1 atm. Aerobic plate counts were determined on samples from each treatment at 3-day intervals over a 12-days storage period. Selected samples were subjected to pH measurements and sensory evaluations during storage.

Microbiological analyses

Each fillet was weighed and 0.1% sterile peptone water was added to make a 1:1 dilution (W/V). A Stomacher Lab Blender 400 (Tekmar, Cincinnati, OH) was used to homogenize specimens for 2 min and then appropriate serial dilutions were made for spiral plating. Aerobic plate counts (APC) were determined by duplicate spiral-platings (Spiral System, Inc., Bethesda, MD) on standard plate count agar (Difco, Detroit, MI) incubated at 30°C for 2 days before counting (Swanson et al., 1992). APC values were expressed as mean $\log_{10}$ CFU/g for 2 fillets/treatment/sampling day. Generation times (GT) during log phase of aerobic bacteria growth were calculated as described elsewhere (Marshall and Schmidt, 1988).

Bifidobacteria were enumerated by pour plating appropriate serial dilutions in 0.1% peptone water with neomycin-paromomycin-nalidixic acid-lithium chloride agar (Teraguchi, 1978; Laroia and Martin, 1991) followed by incubation at 37°C for 48 hr in an anaerobic jar (Gas-Pak; BBL) prior to counting colonies.

Measurement of pH

Fillet surface pH was measured with a standardized pH meter (Accumet^R, Model 50, Fisher) using a surface electrode. Mean pH values were reported as averages of duplicate readings for each duplicate fillet.

Sensory evaluation

Sensory evaluation of samples was performed by an 8-member untrained panel. Uncooked odor and appearance of treated fillets were evaluated every 3 days during storage at 4°C. Samples were warmed to room temperature prior to analyses. Treated samples were compared with a fresh control (fresh fillets were used each analyses day) which was assigned a score of 5. Samples liked less than the control were scored 1 to 4, where 1 = most disliked. Samples liked more than the control were scored 6 to 9, where 9 = most liked. Untreated fillets also were compared with untreated fresh fillets and treated fillets. Two fillets/treatment/sampling day were analyzed.

Statistical analyses

APC, pH, and sensory data were analyzed using ANOVA, and means were separated by the least significant difference test (SAS Institute, Inc., 1992) at $P \leq 0.05$.

RESULTS & DISCUSSION

When a 2.5% culture of *B. infantis* was used alone, little impact on APC was noted with exception of an extension ($P<0.05$) of the lag phase (Fig. 1). Once growth began, the generation time (GT) of aerobic microorganisms on fillets treated with *B. infantis* were not different ($P>0.05$) from control fillets (Fig. 2). Similar ($P>0.05$) extended lag phases and GT were noted for fillets treated with 2.5% cultures of *B. adolescentis* or *B. longum* (results not shown). Results reported are data from *B. infantis* treatments. Lag phase increases of psychrotrophic populations were observed in ground poultry treated with *Lactobacillus plantarum* or *Pediococcus cerevisiae* (Raccach et al., 1979). We assumed that inoculated bifidobacteria did not grow on refrigerated fish fillets and the extension of lag phase may be due to compounds produced during culturing, i.e. acetic and/or lactic acid, that were inhibitory to aerobic spoilage bacteria (Gilliland and Speck, 1975; Ray, 1992). Other reasons for inhibited aerobic growth might include nutrient competition, unfavorable changes in oxidation reduction potential, or production of other antimicrobial compounds.

Fillets treated with 0.5% sodium acetate had APC lower and GT higher ($P<0.05$) than control or bifidobacteria-treated fillets (Fig. 1,2). The microbiological shelf-life (i.e. time to reach APC of 10^7 CFU/g or greater (Ayers, 1960)) of acetate-treated fillets was prolonged by 3 days compared to untreated and bifidobacteria-treated fillets. These results confirmed a previous study showing the effectiveness of sodium acetate as an antimicrobial surface treatment of catfish fillets (Kim and Hearnsberger, 1994).

Combining 2.5% bifidobacteria with 0.3% sodium acetate resulted in lag phase ($P<0.05$) extension and increased GT values ≈8 hr more than controls (results not shown). No differences ($P>0.05$) among species was noted. When 2.5% *B. infantis* was combined with 0.5% sodium acetate, decreases ($P<0.05$) in APC and increases in GT were observed (Fig. 1 & 2). No difference ($P>0.05$) in GT was seen between this treatment and 0.5% sodium acetate alone, resulting in a 3-days microbiological shelf-life extension. *B. infantis* behaved similarly ($P>0.05$) as the other two cultures when combined with 0.5% sodium acetate (results not shown). The combined treatments could increase GT by ≈20 hr compared to the control and 10 hr compared to 2.5% bifidobacteria-0.5% sodium acetate treated fillets. A previous report on sodium acetate combined with potassium sorbate or lactic acid culture attributed antimicrobial effects primarily to sodium acetate (Kim and Hearnsberger, 1994). Our results show that an additive interaction occurred when bifidobacteria were combined with sodium acetate.

Efforts to increase antimicrobial activity of the bifidobacteria culture-sodium acetate treatment proved unsuccessful. Increasing the amount of *B. infantis* culture had no influence ($P>0.05$) on APC (results not shown). Likewise, increasing the time the *B. infantis* culture was incubated prior to application had no influence ($P>0.05$) on APC (results not shown). Reddy et al.

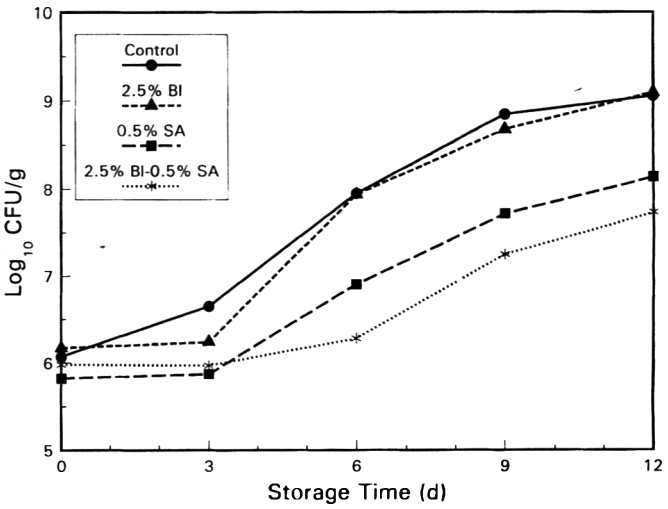


Fig. 1—Aerobic plate counts of catfish fillets treated with *Bifidobacterium infantis* culture (BI) and sodium acetate (SA), either alone or combined, during storage at 4°C.

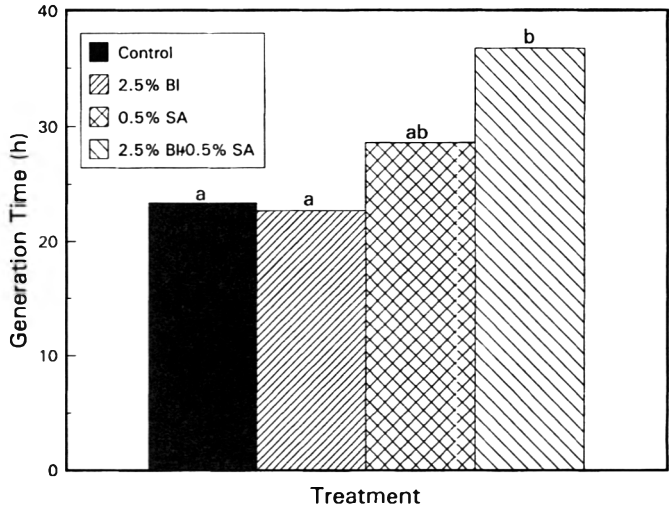


Fig. 2—Generation times of aerobic bacteria at 4°C on catfish fillets treated with *Bifidobacterium infantis* culture (BI) and sodium acetate (SA), either alone or combined. Means with the same letter are not significantly different ($P>0.05$).

(1970) reported that addition of 10% cultures of *Leuconostoc citrovorum* was effective in inhibiting growth of aerobic spoilage bacteria in ground beef stored at 7°C for 7 days. In a later study, Reddy et al. (1983) reported that when cultivation was prolonged from 24 to 48 hr, growth and antibiotic production of *Lactobacillus bulgaricus* in skim milk was enhanced.

Initial pH values of fillets treated with 0.5% sodium acetate and 2.5% bifidobacteria, either alone or combined, were 0.4-0.5 units lower than untreated control fillets (pH 6.8) (results not shown). Other work has shown that sodium acetate-treated catfish fillets did not necessarily have lower pH than controls, but remained inhibitory to growth of aerobes (Kim and Hearnsberger, 1994). Hence, inhibitory results in our study were likely due to changes in pH and to the specific action of sodium acetate.

Sensory scores indicated that treated fillets were in the "liked less" to "typical" category in appearance and odor compared to fresh control fillets (Table 1). Stored untreated control fillets were unacceptable (associated with spoiling fish) by 6 days storage and were discontinued for sensory evaluation. Odor and appearance of fillets treated with 0.5% sodium acetate and 2.5% *B. infantis*, either alone or combined, were the same ($P>0.05$) as fresh fillets up to 6 days. Fillets treated with sodium acetate and bifidobacteria cultures or sodium acetate alone were rated

Table 1—Mean sensory evaluation scores of catfish fillets treated with sodium acetate (SA) anc *Bifidobacterium infantis* culture (BI), during storage at 4°C

	Storage time (d)									
	Odor Score					Appearance Score				
Treatment	0	3	6	9	12	0	3	6	9	12
Fresh Control	5.0[a]	5.0[a]	5.0[ab]	5.0[a]	5.0[a]	5.0[a]	5.0[a]	5.0[a]	5.0[a]	5.0[a]
2.5% BI	4.7[a]	4.8[a]	4.5[b]	3.1[bc]	1.4[c]	4.5[a]	4.5[a]	5.1[a]	3.2[b]	1.5[c]
0.5% SA	4.6[a]	4.4[a]	5.4[a]	3.7[b]	2.2[b]	4.6[a]	4.5[a]	5.3[a]	3.3[b]	2.5[b]
2.5% BI+ 0.5% SA	5.4[a]	5.0[a]	5.0[ab]	3.4[bc]	2.4[b]	4.5[a]	4.8[a]	5.2[a]	3.3[b]	2.6[b]
Untreated Control	5.0[a]	5.1[a]	ND[d]	ND	ND	5.0[a]	5.3[a]	ND	ND	ND

[a-c] Means within the same column with different superscripts are significantly different ($P<0.05$).
[d] ND = Not determined.

higher than fillets with culture alone after 9 days. APC of fillets treated with bifidobacteria cultures alone rapidly increased after 3 days storage, but the fillets were not considered spoiled by the sensory panel until after 6 days.

CONCLUSIONS

COMBINATION of sodium acetate and bifidobacteria cultures was effective in suppressing aerobic spoilage bacteria on catfish fillets and should be considered as a potential method for shelf-life extension. A 3 day shelf-life extension was realized using fillets with high (10^6 CFU/g) initial microbial loads.

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Edible Coatings on Frozen King Salmon: Effect of Whey Protein Isolate and Acetylated Monoglycerides on Moisture Loss and Lipid Oxidation

YVONNE M. STUCHELL and JOHN M. KROCHTA

ABSTRACT

Whey protein isolate (WPI) and acetylated monoglyceride (AMG) coatings were evaluated for effectiveness against moisture loss and lipid oxidation of frozen King salmon. A model gel material was also used to screen coatings for effectiveness against moisture loss. Coatings of low-melting-point AMG used alone or after applying WPI solution or WPI powder were effective in reducing the rate of moisture loss by 42–65% during the first 3 wk of storage. Onset of lipid oxidation was delayed and peak peroxide values were reduced in samples coated with WPI solution/antioxidant overspray or those containing low-melting-point AMGs. No differences in effectiveness were found among the coating treatments.

Key Words: salmon, edible films, whey protein, WPI, lipid oxidation, moisture loss

INTRODUCTION

FROZEN STORAGE OF FISH is an effective means of preservation which prevents or decreases undesirable chemical changes while maintaining many characteristics of fresh fish. However, during long-term storage, fish and fish products may still deteriorate at a much reduced rate. Edible, protective film coatings can lessen or prevent quality changes and prolong storage life in foods such as frozen fish by acting as barriers to control moisture transfer, oxygen uptake and loss of volatile aromas and flavors. Such coatings must have appropriate mechanical and sensory properties. Edible films can be made from proteins, polysaccharides, lipids or a combination of these materials as either bilayers or emulsions (Guilbert, 1986; Kester and Fennema, 1986; Krochta, 1992).

Many types of coating materials have been tested in attempts to maintain quality in frozen foods. Ice glazing has often been used to retard lipid oxidation and moisture loss (Khayat and Schwall, 1983), but brittleness of ice and loss due to sublimation requires that fish be reglazed periodically during frozen storage (Wheaton and Lawson, 1985). Addition of corn syrup solids, cellulose gums and pectinates to glazing solutions has had limited success and acceptance (Ijichi, 1978). Gelling polysaccharides, such as carrageenan and alginate, have been applied as coatings to frozen foods, but were no more effective than ice glazing (Ijichi, 1978; Shaw and Secrist, 1977). Lipid coatings such as acetylated monoglycerides (AMGs) are most frequently used and are expected to provide a good moisture barrier because of their hydrophobicity. Chicken breasts coated with AMG (Myvacet 7-15) and stored at −18°C were comparable after cooking to controls wrapped in polyvinylidene chloride film (Zabik and Dawson, 1963). Hirasa (1991) used AMG (Myvacet 5-07) to coat Silver salmon pieces which were then stored at −10°C for 10 wk, and noted a decrease in relative moisture content of only 2.7% during that time. This coating, however, formed visible cracks during freezing and moisture loss probably occurred through these flaws.

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Proteins have not been investigated as extensively as other biopolymers for edible coatings and films (Krochta, 1992). A 3% aqueous gelatin coating containing antioxidants at 0.15% total concentration provided excellent protection against rancidification in cut poultry when compared to uncoated controls and gelatin coating alone (Klose et al., 1952). Films from whey protein isolate (WPI) have been shown to be excellent oxygen barriers (McHugh and Krochta, 1994). Casein and AMG (Myvacet 5-07) emulsion coatings were used on frozen Silver salmon pieces which were stored at −10°C, and reduced moisture loss when compared to uncoated salmon (Hirasa, 1991). However, the characteristics of multicomponent films have not been fully evaluated at frozen temperatures.

Whey protein has not been tested as a coating under frozen conditions. Previous work with AMG coatings has been with those which are solid at room temperature (≈23°C) and thus they cracked at freezing temperatures. We studied AMGs which are liquid at room temperature and exhibited no visible cracking under freezing conditions. Coatings explored previously for frozen fish were quite thick and opaque and were designed to be easily removed prior to fish analysis after frozen storage (Hirasa, 1991). However, coatings we used were thin, transparent to translucent, and could not be removed readily from the samples. Our objective was to evaluate the effectiveness of various WPI and AMG coatings and compare their effects on rates of moisture loss and lipid oxidation in frozen King salmon.

MATERIALS & METHODS

Materials

Fresh farmed King salmon (*Oncorhynchus tshawytscha*) were purchased from Pacific Fresh Seafood Company (West Sacramento, CA). BiPRO whey protein isolate (WPI) was supplied by Le Sueur Isolates (Le Sueur, MN). Acetylated monoglycerides (AMG) Myvacet 9-08® and Myvacet 9-45®, were obtained from Eastman Chemical Products Inc. (Kingsport, TN). Myvacet 9-08 is a hydrogenated coconut oil, 96% acetylated, with an iodine value of 2 and melting point range of −12 to −14°C. Myvacet 9-45 is a partially hydrogenated soybean oil, 96% acetylated, with an iodine value of 43–53 and melting point range 4–12°C. Kelgum®, a xanthan gum/locust bean gum mixture, was supplied by Kelco (San Diego, CA); and Firm-Tex® starch was supplied by National Starch and Chemical Company (Bridgewater, NJ). L-ascorbic acid, citric acid monohydrate, chloroform, methanol, benzene, and hydrochloric acid were purchased from Fisher Scientific Co. (Pittsburgh, PA). Ammonium thiocyanate, ferrous sulfate heptahydrate and ferric chloride hexahydrate were purchased from Aldrich Chemical Company Inc. (Milwaukee, WI). Barium chloride was obtained from Sigma Chemical Co. (St. Louis, MO). All chemicals used were ACS grade.

Treatments

Ten treatments were tested: Uncoated (control); 10% WPI solution spray; 10% WPI solution spray followed by 5% antioxidant overspray; Myvacet 9-08 AMG spray; Myvacet 9-45 AMG spray; 10% WPI solution spray followed by Myvacet 9-08 overspray; WPI powder dusting followed by Myvacet 9-08 overspray; WPI powder dusting followed by Myvacet 9-45 overspray; 10% WPI:10% Myvacet 9-08 emulsion spray; 10% WPI:10% Myvacet 9-45 emulsion spray.

Table 1—Average weight of coatings applied to fish samples[a]

Treatment	Wt spray or powder coating (g)	Wt overspray coating (g)
Uncoated	—	—
10% WPI spray	1.61 ± 0.46	—
10% WPI spray/Antioxidant overspray	1.27 ± 0.20	0.76 ± 0.17
10% WPI: 10% Myv 9-08 emulsion spray	3.21 ± 0.48	—
10% WPI: 10% Myv 9-45 emulsion spray	2.45 ± 0.26	—
10% WPI spray/Myv 9-08 overspray	1.27 ± 0.18	0.58 ± 0.12
WPI Powder/Myv 9-08 overspray	0.47 ± 0.07	0.99 ± 0.22
WPI Powder/Myv 9-45 overspray	0.45 ± 0.06	0.41 ± 0.21
Myv 9-08 spray	1.00 ± 0.31	—
Myv 9-45 spray	1.78 ± 0.21	—

[a] Average weight of fish samples to which coating was applied, 48g; average exposed surface area, 63 cm^2.

Coating preparation

A 10% (w/w) WPI solution was prepared using 300 g WPI powder and 2700g distilled water, then heating for 20 min in a 90°C water bath and cooling to 5°C with stirring. Both 10% WPI:10% Myvacet 9-08 and 10% WPI:10% Myvacet 9-45 emulsion solutions were made by adding 100g WPI powder to 800 g distilled water, then heating and cooling as with WPI solution. Next, 100g of either Myvacet 9-08 or Myvacet 9-45 was added, and the solution was emulsified for 3 min at 13500 rpm followed by 8 min at 20500 rpm using an Ultra-Turrax T-25 homogenizer with an 10 mm KG probe. The solution was cooled to 5°C prior to application. A 5% antioxidant solution composed of 2.5% each citric and ascorbic acid (w/w in distilled water) was made and cooled to 5°C prior to application.

Preparation of model gel for preliminary tests

A model gel-like material was developed for evaluating effectiveness of frozen coatings in reducing moisture loss. A 1% Kelgum/2% Firm-Tex (w/w in distilled water) solution was made using a Braun (type 4-172) handheld mixer. The solution was heated with stirring to 78°C and held at that temperature for 1 min. Round plastic petri dishes 35 × 10 mm were filled with hot solution and the cover placed on the dish to remove excess material. The dishes were set on a level surface to cool and gel at ambient temperature, then stored at 4°C until coating. At the time of coating, gels were removed from the dishes. Coating was accomplished by dipping each gel into the coating material, allowing excess to drip off, placing the coated gel into a 8 cm hexagonal weighing dish, and freezing at −23°C. Triplicate samples of each coating were tested.

Fish sample preparation

Twenty King salmon (*Oncorhynchus tshawytscha*), 2.75–3.65 kg whole gutted fish, were purchased 3–4 days post slaughter, filleted, skinned, wrapped in plastic, packed in crushed ice and stored at 2°C overnight. Fish fillets were partially frozen prior to trimming to remove outer flesh containing dark muscle, resulting in a sample thickness of 1.25–2.00 cm. Samples (6.3 cm diameter, avg wt 48g) with average exposed surface area (top and side surface) of 63 cm^2 were frozen at −28°C. The WPI powder coating was applied to samples prior to freezing to ensure good adhesion. Protein solution and emulsion coatings were applied as a fine spray after fish freezing using a home garden sprayer. Myvacet and antioxidant oversprays were applied using Crown® Spra-Tool (Fisher Scientific Co., Pittsburgh, PA). Time between fish purchase and sample coating was <38 hr. After coating, samples were stored at −23°C. Table 1 gives the average weight of each spray or powder coating and overspray applied to the fish samples. Dishes of pure ice, AMG coated ice, and AMG were also placed in the freezer as references.

Moisture loss

The total weight change of each sample from time of coating to date of sampling was used to assess weight loss of stored samples. At each sampling time, samples were visually examined for cracks in the coating. Moisture content of each sample was reported as relative moisture content and represents the average of five measurements for each treatment. For comparison among treatments, the average rate of moisture loss over the initial 3-wk period and the 3-thru-8-wk period were calculated.

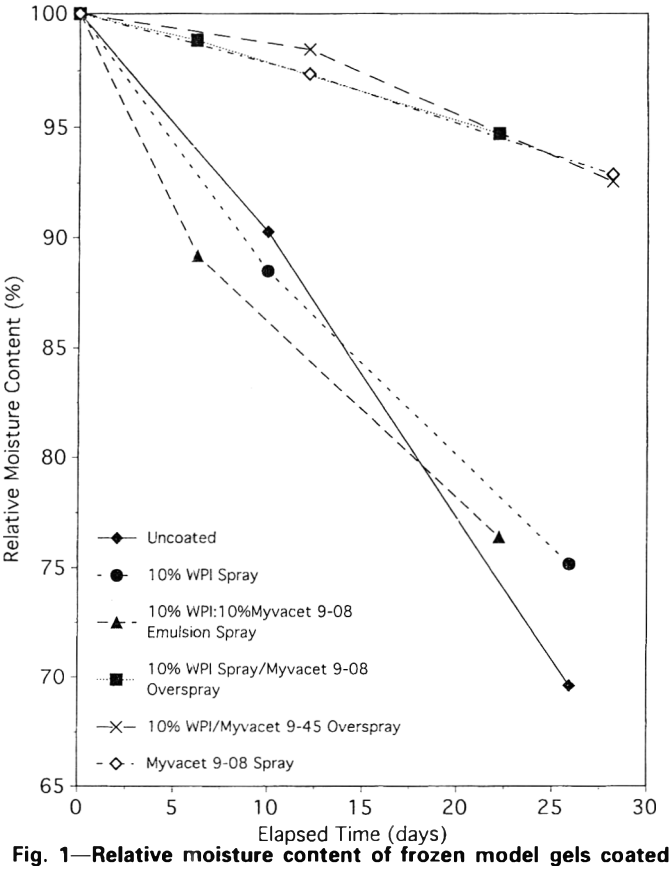


Fig. 1—Relative moisture content of frozen model gels coated with WPI and acetylated monoglyceride edible coatings and stored at −23°C.

Lipid extraction

Lipid was extracted from the fish sample using the method of Bligh and Dyer (1959) with some modifications. About 1/4 of the fish sample with its coating was chopped into small pieces with a sharp knife and placed into a 125 mL high density polyethylene flask. The flask was placed in an ice bowl, 10 mL chloroform and 20 mL methanol were added, and the mixture was homogenized for 2 min at 13500 rpm with an Ultra-Turrax T-25 homogenizer using an 8 mm Kg probe. Next, 10 mL chloroform was added and homogenization was continued for an additional 30 sec. Finally, 10 mL distilled water was added and the mixture was homogenized for 30 sec more. This mixture was divided into two 50 mL centrifuge tubes and centrifuged 30 min at 1500×g using an IEC centrifuge. The chloroform layer was removed and stored at −28°C until testing.

Determination of lipid oxidation

Lipid oxidation was determined using the method of Chapman and Mackay (1949) to determine peroxide value of the sample. The lipid/chloroform mixture was evaporated at 50°C in a water bath with nitrogen blowing over the solution to avoid further oxidation. Aliquots of lipid were weighed accurately, mixed with 9.9 mL benzene:methanol (7:3), and vortexed until completely dissolved. One drop each of ammonium thiocyanate and ferrous chloride solution were added, vortexed, heated at 50°C in a water bath for 2 min, and then cooled quickly to ambient temperature. The absorbance of each solution was measured at 510 nm using a Shimadzu UV-1201 UV-Vis spectrophotometer and used to calculate the peroxide value. Peroxide values represent the average of duplicate measurements for each sample and 5 samples for each treatment.

Statistical analysis

StatView 4.0 was used for all statistical analyses (Abacus Concepts, Berkeley, CA). Analyses of variance and Fisher PLSD multiple comparisons were performed.

Table 2—Average rate of moisture loss as affected by thickness of coatings applied to fish samples

Treatment	Avg total thickness of coatings[a] (mm)	Average rate of moisture loss			
		0–3 wk (g/cm^2 wk)	per mm coating, 0–3 wk (g mm/cm^2 wk)	3–8 wk (g/cm^2 wk)	per mm coating, 3–8 wk (g mm/cm^2 wk)
Uncoated	—	0.019	—	0.015	—
10% WPI spray	1.7	0.025	0.043	0.017	0.029
10% WPI:10% Myv 9-08 emulsion spray	3.4	0.022	0.075	0.017	0.058
10% WPI spray/Myv 9-08 overspray	1.6	0.011	0.018	0.012	0.019
WPI powder/Myv 9-08 overspray	1.8	0.007	0.013	0.010	0.018
Myv 9-08 spray only	1.1	0.014	0.015	0.013	0.014
Uncoated ice control	—	0.087	—	0.087	—
Myv 9-08 coated ice control	1.5	0.005	0.008	0.005	0.008

[a] Average thickness of the coating was calculated from weight of coating(s), total surface area of sample, density of the coating materials at 5–7°C, and assuming uniform coating.

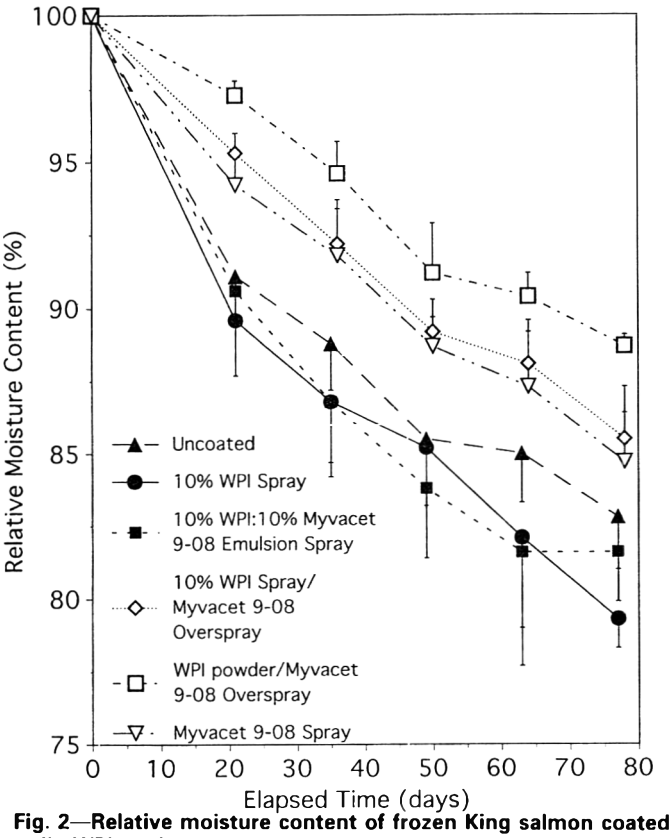


Fig. 2—Relative moisture content of frozen King salmon coated wtih WPI and acetylated monoglyceride edible coatings and stored at −23°C.

RESULTS & DISCUSSION

Model tests

Effects of WPI and acetylated monoglyceride coatings were compared on model gels over a 30-day test period (Fig. 1). The 10% WPI and 10% WPI:10% AMG emulsion coatings had moisture loss curves similar to uncoated gels. Coatings of AMGs and bilayer coatings of 10% WPI/AMG had much slower moisture loss compared to uncoated gels. The 10% WPI and AMG coatings were translucent, while emulsion coatings were milky in appearance.

King salmon moisture loss test

Total thickness of coatings, calculated from weight of the coating(s), total surface areas, density of coating materials at 5–7°C, and assuming uniform coating were compared (Table 2). Effects of WPI and AMG coatings on relative moisture content of King salmon over 11 wks were compared (Fig. 2). Results were similar between the 10% WPI spray and 10% WPI spray/5% antioxidant overspray coatings and between Myvacet 9-08 and Myvacet 9-45 coatings with respect to moisture loss, thus data for antioxidant overspray and Myvacet 9-45 coatings were not included.

Moisture losses of fish samples coated with 10% WPI and 10% WPI:10% AMG emulsion coatings were comparable at all sampling times to uncoated samples. Average rates of moisture loss for these treatments were initially (0–3 wk) higher than those with an AMG spray or overspray, indicating that WPI and WPI emulsion coatings provided little moisture barrier at freezing temperatures (Table 2). Although the WPI emulsion coating was twice as thick as the WPI coating, it was initially (0–3 wk) only slightly more effective at reducing moisture loss. The average rates of moisture loss remained similar among uncoated, 10% WPI and WPI emulsion coated samples during the following 8 wk period, despite coating thickness differences (Table 2). Pham and Willix (1984) found that the desiccated surface layer, which remains behind as an ice front recedes during frozen storage of biological foodstuffs, produced a layer through which moisture must diffuse, causing resistance to mass transfer of moisture. A dessicated surface layer on the salmon formed during long storage times and was probably an additional barrier to moisture loss, resulting in lower rates of moisture loss for uncoated, WPI and emulsion coated fish during the last 8 wk of testing.

Samples coated with AMG spray or overspray had less ($p<0.05$) moisture loss than uncoated samples (Fig. 2). The WPI powder/Myvacet 9-08 overspray was a whitish coating, but had the lowest initial (0-3 wk) moisture loss rate per mm coating thickness (Table 2). This may have been due to a lower moisture coating on the surface of the fish, providing more initial resistance to moisture transfer. In addition, the dry protein coating probably provided a more uniform, less hydrophilic surface, allowing the AMG to bind more effectively to the surface. The 10% WPI spray/Myvacet 9-08 overspray coating, which was translucent, had higher initial moisture loss per mm coating thickness than the Myvacet 9-08 spray alone, which was almost transparent. During the last 8 wk period, as with the 10% WPI and WPI emulsion coatings, differences among treatments decreased with respect to rate of moisture loss (Table 2). The Myvacet 9-08 spray coating had the lowest moisture loss per mm coating thickness over this period.

Thus, edible film coatings consisting of AMGs alone or over protein coatings, were effective in reducing moisture loss. The effectiveness of low melting point AMG coating in slowing sublimation of ice suggested that it may be used to protect ice glazes on frozen fish.

King salmon lipid oxidation test

Peroxide values (PV) of King salmon pieces coated with WPI and/or AMG during the 11 week study were compared (Table 3). PVs of the salmon pieces did not show significant differences until the fifth week of testing. At the fifth week, all coatings reduced the PV ($p<0.10$) compared to uncoated samples. The WPI powder/Myvacet 9-08 overspray coating had the lowest

Table 3—Peroxide values* (Meq/kg) of frozen King salmon pieces coated with WPI and acetylated monoglyceride coatings and stored at −23°C

Treatment	Elapsed time (days)				
	21	35	49	63	77
Uncoated	1.67 ± 0.79[a]	3.69 ± 0.91[a]	2.70 ± 0.50[abcd]	2.34 ± 0.95[ab]	2.56 ± 0.39[ab]
10% WPI spray	1.27 ± 0.39[a]	2.93 ± 1.56[bc]	2.73 ± 0.20[abc]	1.88 ± 0.55[abc]	2.36 ± 0.92[abc]
10% WPI spray/antioxidant overspray	1.54 ± 0.54[a]	1.65 ± 0.61[cde]	2.65 ± 0 96[abcde]	1.74 ± 0.58[abc]	1.16 ± 0.32[cd]
10% WPI:10% Myv 9-08 emulsion	1.48 ± 0.69[a]	1.50 ± 0.34[cde]	2.16 ± 0.48[bcde]	1.69 ± 0.51[abc]	1.84 ± 0.60[bcd]
10% WPI spray/Myv 9-08 overspray	1.69 ± 0.60[a]	2.19 ± 0.60[bc]	2.45 ± 0 51[abcde]	1.57 ± 0.40[bc]	1.44 ± 0.55[cd]
WPI Powder/Myv 9-08 overspray	1.82 ± 0.97[a]	1.39 ± 0.51[de]	2.45 ± 0 72[abcde]	1.98 ± 0.49[abc]	1.81 ± 0.16[bcd]
Myv 9-08 spray	1.19 ± 0.32[a]	1.70 ± 0.22[cde]	2.12 ± 0 94[cde]	1.73 ± 0.42[abc]	1.69 ± 1.04[bcd]

* Initial peroxide value of salmon pieces was 1.34 ± 0.31 Meq/kg. Statistical analysis for the effect of treatment was performed separately for each elapsed time using Fisher's PLSD multiple comparison method.

[a-e] Treatments with the same letter within each elapsed time are not statistically different ($p \leq 0.10$).

PV. The order of effectiveness of remaining coatings was WPI: Myvacet 9-08 emulsion, WPI spray/antioxidant spray, Myvacet 9-08 spray only, WPI spray/Myvacet 9-08 overspray, with the WPI spray only coating having the greatest PV. At the sampling times of 7, 9, and 11 wk, difference in peroxide values between uncoated samples and the treatments was not as apparent, but almost all treatments were lower than the uncoated controls. In addition, the maximum peak in the PV was reduced and delayed 2 wk for coatings containing antioxidant or AMGs. This suggested that the coatings were effective in slowing diffusion of oxygen through the coating and reducing availability of oxygen to the fish surface. This may also be partially due to the decreased rate of moisture loss with AMG coatings. Banks and Hardy (1965) showed that greater drying loss resulted in higher peroxide values in the fat of 3.2 kg blocks of herrings stored for 6 mo. at −20°C.

Peroxide value is useful during the initial stages of lipid oxidation (Gray, 1978). In fish oils, the rate of hydroperoxide formation greatly exceeds its decomposition during early stages of lipid oxidation and correlates fairly well with oxidation (Smith et al., 1972). Decreases in peroxide values with time have been noted by other researchers. Awad et al. (1968) measured peroxide values in bovine muscle stored in 200g lots at −4°C for up to 8 wk. They found that the PV of beef decreased after 2 wk frozen storage and suggested that this was due to peroxide decomposition or interaction with protein. Noble (1976) measured PV of ground, deboned chicken meat stored in 0.9 kg lots at −23°C for up to 12 wks and found that the PV peaked at 6 wks, then decreased almost to original levels. Hirasa (1991) reported that the peroxide value correlated best with lipid oxidation in salmon when compared to thiobarbituric acid value and headspace gas chromatographic analysis for pentane.

The rate of lipid oxidation in fatty fish such as salmon is affected by several factors. The lipid content and composition of fish flesh can vary greatly depending on age, maturity, size of fish, and the position from where the sample was taken (i.e., head to tail, back or belly flap, dark or light meat) (Stansby and Olcott, 1963; Ackman, 1979; Flick et al., 1992). Fish such as salmon contain a wide variety of types of lipids, some highly unsaturated and highly reactive. The disposition of lipid within the tissue would also affect its rate of oxidation, with the dark muscled flesh tending to oxidize more rapidly than light flesh (Hardy, 1979). Nonlipid components, such as metals and tocopherols, can also affect lipid oxidation rates (Hardy, 1979). Oxidation increases when dehydration occurs (Banks, 1952). Hiremath (1973) showed that the main reason for oxidation of fats in frozen fish was dehydration of the tissue during storage and exposure to atmospheric oxygen. The effect of water activity on lipid oxidation is complex (Nelson and Labuza, 1992). Such complexity suggests that model systems with lipid components are needed to test the effects of edible coatings under frozen conditions.

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Abalone (*Hariltis discus*): Seasonal Variations in Chemical Composition and Textural Properties

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ABSTRACT

Seasonal changes in adenosine 5'-triphosphate, total free amino acids, and total oligopeptides in abalone meat were analyzed. Levels were higher in summer and lower in winter. In both seasons, the most abundant free amino acid was taurine followed by arginine, glycine, glutamine, and glutamic acid. The largest peptide-bond amino acid was glutamic acid + glutamine. The collagen content decreased in summer and increased in winter. The breaking stress values of the meat were low in summer and high in winter, indicating that summer abalone would be more tender. Scanning electron microscopy revealed the collagen network was more compact in winter.

Key Words: abalone, texture, seasonal variations, amino acids, collagen

INTRODUCTION

CHEMICAL COMPOSITION and physical properties of fish meat show seasonal variations, causing variations in texture (Dunajuski, 1979). Seasonal variations in lipid content of Atlantic mackerel (Leu et al., 1981) and yellowtail meats (Shimizu et al., 1973) and changes in pH (Love, 1979) have been reported. Seasonal variations in free amino acids of seafood have also been reported on levels of glutamic acid, glycine, and taurine in lemon sole (Jones, 1959). According to Hughes (1964), only histidine in herring varied throughout the year but no seasonal variation occurred in other free amino acids. Annual cycles of extractive nitrogen, most free amino acids and nucleotides were reported in ascidian (Watanabe et al., 1985).

In Japan, there are customary best and worst seasons for eating fish or shellfish, depending on species. Generally, the best season coincides with the time when the lipid content of fish becomes highest before spawning. Abalone is a very popular food throughout the world, eaten both raw and cooked in Japan. Customarily, summer is the best season and winter is the worst for abalone meat. The reputation of abalone may not be related to lipid content which is only 0.4% (Standard Tables of Food Composition in Japan 1982). Little study has been reported on whether chemical components in abalone show seasonal variations. Only Watanabe et al. (1992) determined the contents of adenosine 5'-triphosphate-related compounds, glycogen, organic acids, and free amino acids analyzing natural live abalone at 2 or 3 mo intervals. From their results, abalone collected in September was highest in almost all compounds. Changes in extractive nitrogen content (Takayama et al., 1970) and glycogen content (Webber, 1970) have also been reported.

Texture is another important factor that determines the preference for abalone and its seasonal variation should be studied in detail. Takayama et al. (1970) reported the hydroxyproline content of abalone was low between May and August and high between October and December, but they did not correlate this with physical properties. Oleaechea et al. (1993) studied seasonal variations in collagen content, although their results were based on small sample sizes.

Our objective was to determine changes in chemical composition and texture of abalone muscle in relation to seasonal variations.

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MATERIALS & METHODS

Materials

Abalone, (*Haliotis discus*), were collected from a coastal area in the Pacific Ocean near the Miura Peninsula and reared for at least 2 mo at the Kanagawa Prefectural Fisheries Experimental Station all year round. They were fed brown macroalga of sea trumpet, (*Eisenia bicyclis* and *Echlonia cava*), which are their dominant foods in the natural habitat. Live specimens were transported to the laboratory. The middle part of the adductor muscle of three individuals was harvested in February, April, June, July, August, October, November and December and used to analyze chemical composition and textural properties. The total weight of the samples including shell was 357.7 ± 24.9g. Triplicate experiments were done to provide mean values.

Proximate composition

Moisture was measured by drying muscle tissues in moisture plates at 100-105°C to constant weight. Crude protein was determined by the micro-Kjeldahl method and crude lipid by the Bligh-Dyer method (Bligh and Dyer, 1959). To determine ash, the sample was heated over a gas burner for 2 hrs and in a muffle oven at 550°C to constant weight.

Minerals

Sodium, potassium, calcium, and magnesium were measured by an atomic absorption method (Japanese Society for Food Science and Technology, 1982) using Shimadzu atomic absorption spectrophotometer AA-660 (Shimadzu Co.Tokyo).

Adenosine 5'-triphosphate (ATP) and related compounds

Abalone meat (5g) was homogenized with 10% perchloric acid (10 mL) using a Polytrone homogenizer (Kinematica Co., Tokyo). The homogenate was centrifuged at 10,000 × *g* for 15 min. The precipitate was re-extracted with 5% perchloric acid (10 mL) and centrifuged. Both supernatants were combined, adjusted to pH 7.0 with KOH and made up to 25 mL. The experiment was conducted at < 4°C. ATP and related compounds were analyzed using Shimadzu HPLC-10 A (Shimadzu Co.Tokyo) with Asahipak GS-320 H column (Asahi-Kasei Co.Tokyo) under the following conditions: mobile phase, a mixture of 200 mM NaH_2PO_4 and 200 mM H_3PO_4 (pH 3.0); flow rate, 1 mL/min; absorbance at 260 nm.

Free amino acids and oligopeptides

Abalone meat (5g) was homogenized with 10% trichloroacetic acid (TCA) (10 mL), followed by centrifugation at 10,000 × *g*. The precipitate was re-extracted with 5% TCA (10 mL). The supernatants were combined and made up to 25 mL. Free amino acids were determined at 440 nm and 570 nm using Hitachi amino acid analyzer 835 S (Hitachi Co. Tokyo) in the same procedure as previously described (Hatae et al., 1991). Total amino acid contents were determined after hydrolysis with 6N HCl for 24 hrs at 110°C; peptide-bond amino acids were determined by subtraction.

Table 1—Seasonal variation in chemical components and textural properties of abalone meat

Month	Moisture (%)	Protein (%)	Lipid (%)	Ash (%)	Total free amino acid (%)	Total peptide bond amino acid (%)	Collagen (% of protein)	Water-holding capacity (%)	Breaking stress ($\times 10^4$ Pa)
February	81.2±2.0	14.5±1.5	0.76±0.16	1.79±0.42	1.71±0.27	0.11±0.07	26.4±8.4	64.7±9.0	4.20±1.18
April	78.8±0.7	16.3±0.3	0.35±0.15	1.64±0.12	2.33±0.29	0.14±0.02	20.2±0.5	80.3±5.5	3.57±0.71
June	74.3±3.1	16.3±1.0	0.26±0.04	1.46±0.12	2.70±0.28	0.36±0.08	13.7±2.2	89.9±6.9	2.97±0.89
July	73.7±3.0	18.0±0.7	0.32±0.16	1.27±0.24	2.15±0.03	0.25±0.11	9.3±2.2	92.4±3.1	1.92±0.39
August	72.4±0.5	18.4±0.7	0.41±0.04	1.11±0.02	2.13±0.06	0.18±0.60	8.9±2.4	93.6±0.9	2.00±0.20
October	79.0±2.4	16.8±0.5	0.37±0.02	1.56±0.16	2.18±0.26	0.12±0.02	26.4±3.0	78.3±3.1	3.70±0.11
November	82.1±2.1	14.2±1.9	0.93±0.08	1.78±0.15	1.48±0.18	0.03±0.00	30.8±0.9	43.9±5.7	4.98±0.78
December	81.5±1.4	15.2±1.1	0.90±0.11	1.92±0.03	1.72±0.17	0.04±0.04	30.6±1.9	32.3±6.3	5.00±0.37

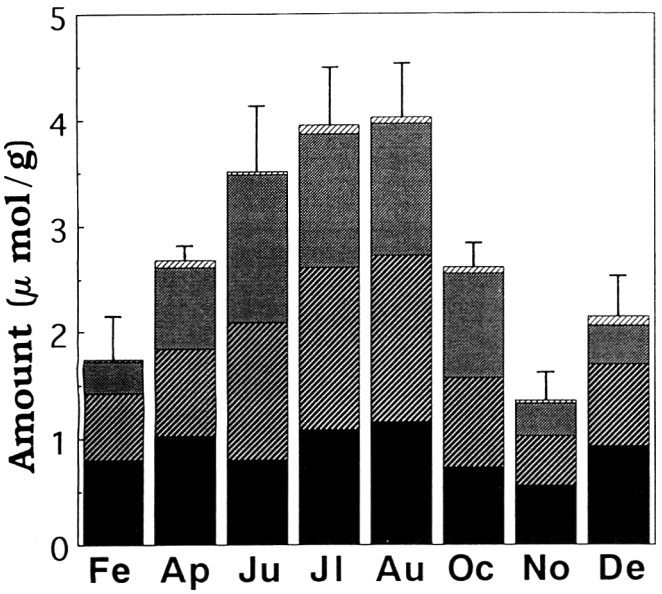


Fig.1—Changes in levels of ATP and ATP related compounds. ■ : ATP, ▨ : ADP, ▧ : AMP, □ : IMP. Fe: February, Ap: April, Ju: June, Jl: July, Au: August, Oc: October, No: November, De: December. Brackets indicate standard deviations.

Collagen

Abalone meat (0.1 g) was hydrolyzed with 6 N HCl (1 mL) at 130°C for 3 hrs and hydroxyproline content was determined using Hitachi amino acid analyzer 835 S. Collagen was calculated on the assumption that the hydroxyproline content in abalone collagen was 10.2% by weight (Kimura and Kubota, 1986).

Textural properties

Abalone meat was sliced into 4 mm thick pieces and subjected to force exerted by a cylindrical plunger of 2 mm diameter at a rate of 0.5 mm/sec using a Rheometer RE-3305 (Yamaden Co., Tokyo). From the time-force curve, the breaking stress (P Pa) was calculated by

$$P = (B \times 98)/S$$

where B is maximum force (g), S is base area of the plunger (cm^2), and 98 is acceleration of gravity (Pa). Five grams abalone meat pieces were centrifuged at 28,000 × g for 30 min. The water holding capacity (WHC) was calculated from the ratio of the weight after centrifugation to that before centrifugation (Hatae et al., 1984).

Scanning electron microscopy (SEM)

Pieces of January and July abalone were prefixed by Karnovsky solution (Karnovsky et al., 1965) and soaked in 2 N NaOH for 3 or 4 days according to Ando et al. (1992). Then, they were postfixed by 2% tannic acid and 1% osmium tetroxide, dehydrated by a series of ethanol treatments using a critical point dryer HCP-2 (Hitachi Co., Tokyo). These samples were analyzed with a SEM JSM-T 220 (JEOL) after ion spattering.

Statistical analysis

Analysis of variance, cluster analysis, and principal component analysis were performed by SYSTAT/SYGRAPH (1992).

RESULTS & DISCUSSION

Chemical composition

Seasonal variations were found in the major constituents of abalone; 72.3–82.1% moisture, 14.2-18.4% crude protein, 0.26–0.93% lipid, and 1.11–1.29% ash (Table 1). Protein content was slightly higher ($p < 0.05$) and moisture was slightly lower ($p < 0.01$) in summer. Carbohydrate, by difference, was in a range 0.5–7.7%; the highest levels were observed between June and August and the lowest levels in October. Since the spawning of abalone is in November and December in the harvesting area, this result agreed with Webber (1970) who had reported that glycogen had decreased before spawning.

Mineral levels were: sodium 449.9 ± 102.3, potassium 131.3 ± 37.8, calcium 33.8 ± 11.0, and magnesium 28.2 ± 5.6 (mg/100g). Sodium was slightly lower in summer. Sodium and potassium contribute to the taste of snow crab (Hayashi et al., 1981) and scallop (Watanabe et al., 1990).

ATP and related compounds

Total amount of these compounds was 1.4–4.0 μmol/g meat. This was almost the same as reported by Nishita et al. (1970) and Arai and Saito (1961). The total amount of ATP and ATP-related compounds of ascidian (Watanabe et al., 1985) and abalone (Watanabe et al., 1992) increased in summer and decreased in winter. In our study, the same trend was observed (Fig. 1).

Nucleic acid-related compounds are metabolized as ATP→, adenosine 5'-diphosphate (ADP)→, adenosine 5'-monophosphate (AMP)→, inosine 5'-monophosphate(IMP)→, inosine→, hypoxanthine in fish and mammals and ATP→, ADP→, AMP→, adenosine→, inosine→, hypoxanthine in marine invertebrate animals (Saito and Arai, 1957; Arai, 1966a, 1966b). Abalone follows both courses of metabolism (Arai, 1966b). However, the activities of AMP-deaminase and adenosine-deaminase are low (Arai, 1961, 1966a). This suggests that abalone accumulates mainly AMP instead of IMP or adenosine (Olley and Thrower, 1977). Though AMP does not have taste, it shows an "umami" taste by synergism with glutamic acid (Konosu, 1973). The large content of AMP in summer was assumed to be one of the reasons why summer is customarily the best season for eating abalone in Japanese cookbooks (Umezao and Tsuji, 1975).

Free amino acids and oligopeptides

A seasonal variation of total free amino acids was found (Table 1) together with the total amino acids in oligopeptides. The highest free amino acid was taurine (Fig. 2). Arginine, glycine, glutamine and glutamic acid were also dominant components. Taurine was higher in summer abalone than in other seasons. Each of the other amino acids was high in April through June

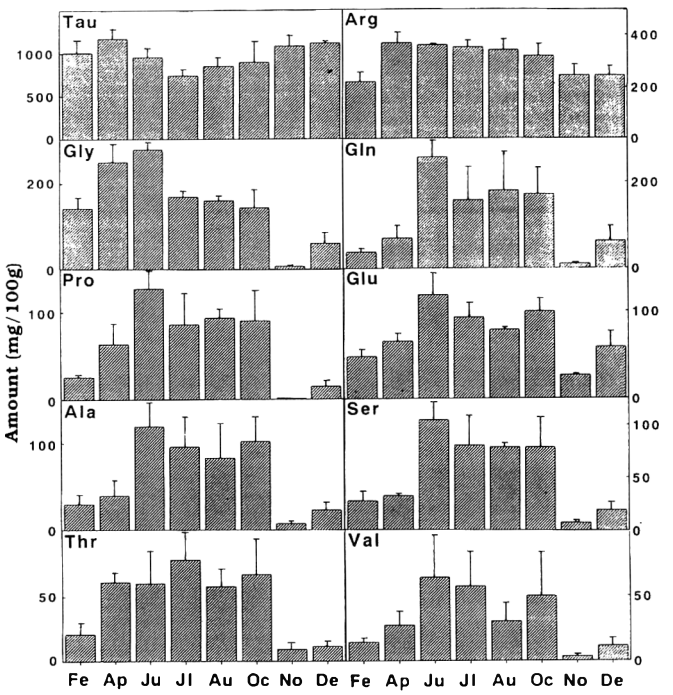


Fig.2—Seasonal variations of major free amino acids. Symbols of the month are the same as in Fig. 1. Brackets indicate standard deviations.

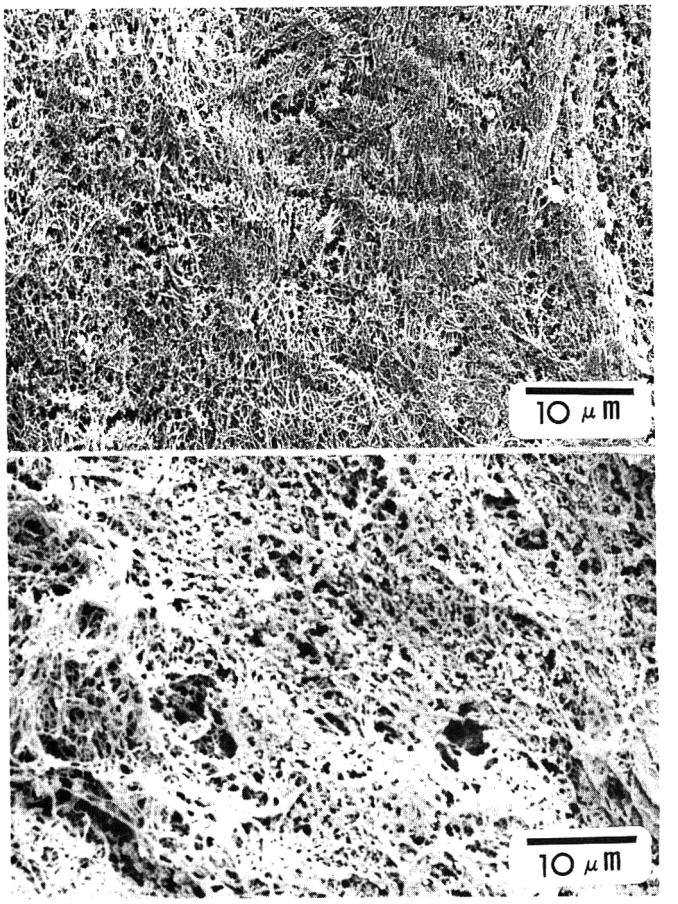


Fig.3—Scanning electron micrographs of summer (lower) and winter abalone meat.

and low in November and December. This amino acid composition was almost the same as results of Konosu and Maeda (1961) who analyzed abalone in May, and those of Watanabe et al. (1992). Konosu (1973) also surveyed the components characterizing abalone flavor by an omission test, describing that glutamic acid gave an "umami" taste and a special abalone flavor and glycine made up the sweet taste. Neither taurine nor arginine contributed abalone flavor though their amounts were large. When AMP was missing, "umami" taste decreased.

Total amount of peptide-bond amino acids was also the highest in June and lowest in November (Table 1). The highest amino acids in the peptides were glutamine + glutamic acid (101.5 ± 87.7 mg %), then glycine (10.3 ± 5.1), asparagine + aspartic acid (4.5 ± 2.5), proline (4.1 ± 3.0) and arginine (3.0 ± 2.6) in decreasing order. The levels of each amino acid decreased or increased in the same order as did total amino acids. Oligopeptides containing glutamic acid, glycine and/or alanine have flavor potentiating activity (Noguchi et al., 1975; Kato et al., 1989). The improved taste of abalone in June may be related to the higher levels of glutamic acid and glycine and their related peptides.

Collagen content and texture

A seasonal variation of collagen content agreed with the breaking stress and water holding capacity (Table 1). Collagen content related to firmness of raw fish meat (Sato et al., 1986; Hatae et al., 1986) and the same relation could be seen here. The abalone was tender and its collagen content was lowest in summer, whereas it was tough and had highest collagen in winter. These changes in muscle structure were clearly observed by SEM (Fig. 3). In the winter abalone, fine fiber networks were more compact compared to summer abalone. Since specimens were treated with NaOH, muscle fiber had been removed, so these fiber networks were assumed to be collagen fibers. Ando et al. (1992) observed the same fibrous structure in globe fish and regarded them as collagen fibers. This structural difference may affect textural properties when abalone meat is masticated. We hypothesized that the texture of winter abalone is not preferable because it is too tough and has too much drip because of decreased WHC.

The reason for the large change in collagen content in the muscle from 9.34% (of muscle protein) in July to 30.82% in November needs further study. Collagen in the abalone meat may be important in energy storage and may have some effect on muscle metabolism before the spawning season, to make the gonads grow. An extraordinary large growth of gonad index in abalone in spawning season has been reported (Webber, 1970). Thus, abalone needs much energy around the spawning season. Abalone, different from fish, contains only small amounts of lipid, therefore, it is unlikely to store lipid for energy. We assumed if abalone stored energy in muscle, storage of collagen might be reasonably expected because collagen was mainly composed of non-essential amino acids. Synthesis and decomposition might occur largely around spawning season. In summer, such turnover might not be so active. Glycine and proline found in peptides were assumed to result from decomposition of collagen. However, this hypothesis was not proved. The seasonal variation was the same as that proved by Olaechea et al. (1993).

Seasonal variation in chemical components and texture

To show graphically and make explicit the seasonal variation in chemical components and texture a cluster analysis was run (Fig. 4). The summer abalone values clustered different from those of winter abalone. Accordingly, a principal component analysis was tried. Loading coefficients, eigenvalues and proportions are shown in Table 2. The cumulative proportion of the first, second and third principal component was 82%. Therefore, more than 80% of variations could be explained using these three components, indicating that they could adequately explain the characteristics of chemical composition and texture. The first principal component is high in protein, ADP, AMP, free amino acids, and peptide-bond amino acids, and low in

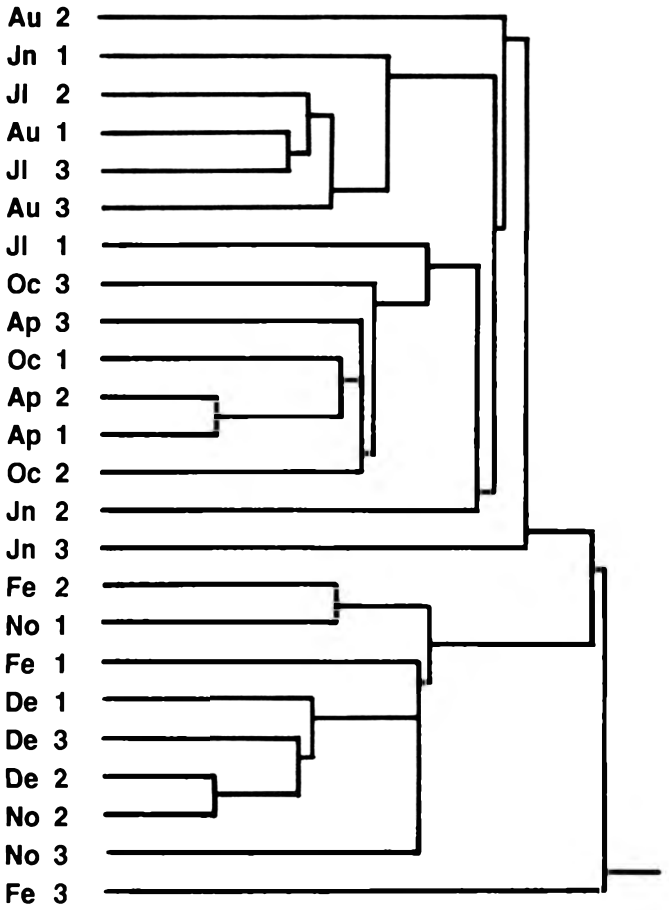


Fig 4—Cluster analysis of abalone samples. Symbols denote: Feb 1-3, Three samples harvested in February; Ap 1-3, Harvested in April; Jn 1-3, Harvested in June; Jl 1-3, Harvested in July; Au 1-3, Harvested in August; Oc 1-3, Harvested in October; No 1-3, Harvested in November; De 1-3, Harvested in December.

Table 2—Principal component analysis of the data from abalone meat

	First	Second	Third
Component			
Moisture	−0.932	0.042	0.105
Ash	−0.852	0.322	−0.011
Lipid	−0.793	−0.309	−0.176
Protein	0.810	−0.327	−0.028
Collagen	−0.844	0.103	0.108
Water holding capacity	0.781	0.189	0.097
Component loadings			
ATP	0.624	−0.579	−0.383
ADP	0.917	−0.128	−0.207
AMP	0.843	0.310	0.238
IMP	0.134	−0.798	0.474
Breaking stress	−0.759	−0.342	0.436
Free amino acid	0.810	0.158	0.378
Oligopeptide	0.843	0.251	0.209
Eigenvalues	8.110	1.644	0.911
Proportion	62.38	12.65	7.04
Cumulative proportion	62.38	75.03	82.04

moisture, collagen, and breaking stress. This could be interpreted as the axis which demonstrates the degree of tastiness. The second principal component was high in AMP and ash, and low in IMP, ATP, and breaking stress, so this could be demonstrating the degree of some index of freshness.

All abalone samples were plotted using three dimensional axes (Fig. 5). Summer abalone, harvested in June (b, Fig. 5), July (c, Fig. 5), and August (d, Fig. 5) were located together in the upper section (Fig. 5). Winter abalone, harvested in November (f, Fig. 5), December (g, Fig. 5), and February (h, Fig. 5) are in the lower section. The spring and autumn abalone groups, and they linked the two groups to make a circle. From this figure we can see seasonal variations of abalone including chemical components and texture.

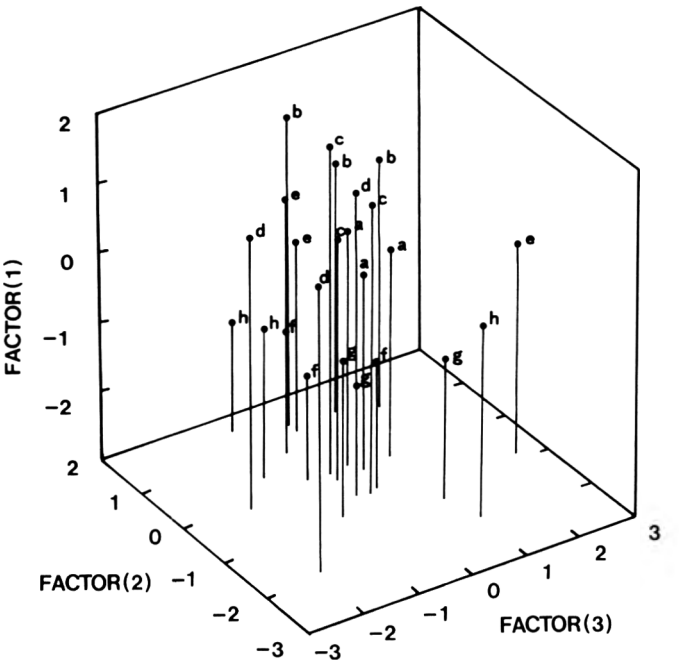


Fig 5—Three dimensional chart of seasonal variation of abalone meat. Symbols are: a, April; b, June; c, July; d, August; e, October; f, November; g, December; h, February.

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—Continued on page 39

Microencapsulation of Squid Oil with Hydrophilic Macromolecules for Oxidative and Thermal Stabilization

CHIH-CHENG LIN, SHAN-YANG LIN, AND LUCY SUN HWANG

ABSTRACT

The oxidative and thermal stabilities of unencapsulated and encapsulated squid oils were compared by polyene ratio, DSC-FTIR and pressure DSC. Wall material containing gelatin, caseinate and maltodextrin provided optimal protection against oxidation. Stability was improved when lecithin and Avicel were part of the wall material. The synergistic effects of lecithin with naturally occurring α-tocopherol and the compact structures of microcapsules enhanced stability. Stability of squid oil microcapsules decreased when α-tocopherol was removed by alumina chromatography. Both DSC-FTIR and pressure DSC were suitable for evaluating oxidative and thermal stability of encapsulated oil products.

Key Words: squid oil, microencapsulation, macromolecules, oxidative/thermal stabilities

INTRODUCTION

DIETARY INTAKE of n-3 polyunsaturated fatty acids (PUFA) has been linked with blood cell anti-aggregation and reduced risk of coronary heart disease (Hearn et al., 1987). Squid oil contains considerable amounts of n-3 PUFA and may be a useful dietary supplement. However, its oxygen sensitivity limits its usage in foods (Lin and Hwang, 1993). Moreover, degradation products of PUFA oxidation not only give rise to off-flavor but excess ingestion could be hazardous to health (Kinsella, 1986). Stabilization of squid oil for potential food use may be beneficial and requires research.

Microencapsulation can effectively protect materials from environmental influences (Dziezak, 1986). The wall or shell of the microcapsule may provide a barrier against adverse influences (Dziezak, 1988). Macromolecules, e.g. protein, gum, and modified starch, have been used as wall materials (Grave, 1972; Blenford, 1986). Proteins were found to be protective against lipid peroxidation in such powdered systems (Hayes et al., 1977; Iwami et al., 1987, 1988; Taguchi et al., 1988). In many cases, wall materials containing protein and carbohydrate produced free flowing products with good rehydration properties (Bakan, 1973; Hibino and Fukuda, 1985). Ono and Aoyama (1979) improved oxidative stability of oils by encapsulation into a protein-carbohydrate matrix.

Our objective was to stabilize squid oil by microencapsulation to provide a potential dietary supplement for n-3 fatty acids. The effect of hydrophilic macromolecules as wall materials in protecting squid oil microcapsules from oxygen was examined and the thermal stability of microcapsules was determined.

MATERIALS & METHODS

Materials

Crude squid oil was obtained from Fon-I Co. (Kaoshiung, Taiwan, ROC). Wall materials were gelatin (200 bloom, Gem Font Co. Taipei, ROC), sodium caseinate (New Zealand Dairy Board, Wellington, New Zealand), and maltodextrin (American Maize Products Co., IN). Egg yolk lecithin was purchased from Sigma (St. Louis, MO) and sodium carboxymethyl cellulose (Avicel RC-591) was obtained from FMC Co. (Philadelphia, PA).

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Microencapsulation

Crude squid oil was alkaline refined with 3N sodium hydroxide and bleached with 20%(w/w) active clay using the procedures of Lin and Hwang (1993). It was deodorized by molecular distillation (KDL-4, Leybold-Heracus, Germany) under the following conditions: feed rate, 10mL/min; operating temp, 185°C; operating pressure, $1-3 \times 10^{-1}$ mbar. α-Tocopherol in the squid oil was removed by alumina chromatography, as described by Yoshida et al. (1975). The molecular distilled or alumina purified squid oil was first emulsified into an aqueous solution of water-soluble macromolecules (composition, Table 1) by homogenizing with a Polytron homogenizer (Kinematica, Japan) at 10,000 rpm for 1 min. The emulsion was fed into the spray dryer (GA32, Yamamoto Sci. Co. Ltd., Japan) with a peristaltic pump at 5 mL/min, and spray atomized into the drying chamber. The spray dryer was operated under the following conditions: inlet temperature, 120°C; outlet temperature, 70°C; flow rate of drying air, 0.4 m^3/min; and pressure of atomizing air, 1.4 kg/cm^2.

Scanning electron microscopy

The microcapsules were coated with a gold layer using an ion sputter (Hitachi E101, Japan). The shape, surface topography and inner structure of the microcapsules were observed with a scanning electron microscope (Hitachi S-2400, Japan).

Determination of oil content

A known amount of microcapsule was dissolved or suspended in 80°C hot distilled water, and the total oil content of the microcapsules was determined by an enzymatic colorimetric method (McGowan et al., 1983). Encapsulated oil was determined by the same method after removing the unencapsulated surface oil by washing 3 times with n-hexane. The unencapsulated oil content was the difference between total oil content and encapsulated oil content.

Oxidative stability

Individual 1 g portions of squid oil microcapsules were stored at 50°C in a 20 mL open amber glass sample vial (1.75 cm i.d. × 5.4 cm) and analyzed for fatty acid contents at different time intervals. Fatty acid contents were determined by GC as described by Lin and Hwang (1993). The degree of oxidation was expressed as the polyene ratio:

$$\text{Polyene ratio} = \frac{[\text{EPA}] + [\text{DHA}]}{[16{:}0]} \times 100$$

where [EPA] is the eicosapentaenoic acid (EPA) content, [DHA] is the docosahexaenoic acid (DHA) content and [16:0] is the palmitic acid content.

Thermal stability

Thermal stability was determined with a pressure differential scanning calorimeter (PDSC, Dupont 910, USA) at a scanning rate of 5°C/min, and 13.4 kg/cm^2 in nitrogen. Microscopic structural changes associated with heating the microencapsulated oil and pure oil were evaluated using a system which combined the FTIR microscope spectrometer (Jasco, Japan) and a DSC (Mettler, FP-84, Switzerland). The IR spectra of oil and microcapsules were measured.

Table 1—Formulations for preparing squid oil microcapsules

Code	Component (g)				
	Oil	Gelatin	Caseinate	Maltodextrin	Lecithin + Avicel
GM	30	30		30	
CM	30		30	30	
GCM	30	20	20	20	
GCM20	20	20	20	20	
AGCM	30[a]	20	20	20	
GCML	30	20	20	20	4 - 1

[a] Molecular distilled oil was replaced by alumina-purified oil.

Table 2—Oil content of microcapsule and encapsulating efficiency

Code	Oil content (mg/g)		Encapsulating[a] efficiency
	Encapsulated	Unencapsulated	
GM	127.5	63.8	38.25
CM	94.8	37.4	28.44
GCM	238.9	30.4	71.67
GCM20	251.3	25.1	75.39
GCML	304.2	9.7	91.26

[a] Encapsulated oil/original oil content in the formulation (Table 1).

Statistical analyses

Results were reported as averages of two independent experiments. Each treatment was analyzed by one-way analysis of variance (ANOVA). Duncan's multiple range test was applied to determine significance of differences between means.

RESULTS & DISCUSSION

THE MICROCAPSULES with highly encapsulated oils are protected against oxidation, and exhibit long shelf lives (Matsuno and Imagi, 1991). Microcapsules with protein-carbohydrate matrix had good protection (Ono and Aoyama, 1979). In our study, the encapsulating abilities of the different formulations of wall materials were evaluated by the amounts of oil which could be encapsulated. Microcapsules with gelatin wall materials contained more encapsulated oil compared to caseinate wall materials (Table 2). Microcapsules GM had more unencapsulated oil; however, amount of oil encapsulated increased when caseinate and gelatin were both present (GCM and GCM20). GCM20 had more oil in the microcapsules than GCM although less oil was present in the emulsion. This indicated that less core material would result in better micro-encapsulation when the same amounts of wall material were used.

Table 3—Polyene ration of squid oil microcapsules stored at 50°C in the dark

Code	Polyene ratio[g]		
	2 wk	4 wk	6 wk
GM	55.3[a]	16.0[a]	3.8[a]
CM	9.4[b]	11.2[b]	3.9[a]
GCM	118.7[c]	33.7[c]	3.3[a]
GCM20	149.2[d]	37.3[c]	3.4[a]
AGCM	45.8[a]	2.3[d]	nd[f]
GCML	180.4[e]	146.7[e]	146.3[b]

[a-e] Values with different superscript within a column are significantly different ($P < 0.05$).

[f] nd means nondetectable.

[g] Polyene ratio of powdered fish oil freshly prepared was 187.2.

Polyene ratio of squid oil microcapsules stored at 50°C (Table 3) showed a sharp decrease between 2 to 4 wks storage for all except GCML. The combination of gelatin, caseinate and maltodextrin as wall material without lecithin and Avicel provided protection from degradation for only 2 wk. Although gelatin has good encapsulating ability (Taylor, 1983), it provided little protection against oxidation. When squid oil was stripped of α-tocopherol by alumina chromatography and microencapsulated (AGCM), polyene ratio was reduced considerably compared to GCM after 2 wk, implying that α-tocopherol was a necessary antioxidant in squid oil. When lecithin and Avicel were included in the formulation (GCML), the oxidative stability increased (Table 3). The polyene ratio value of microcapsule GCML remained > 146.7 for 6 wk. This might be because lecithin was a synergist with α-tocopherol in preventing lipid oxidation (Lin et al., 1993). In addition, lecithin acted as a emulsifier and Avicel acted as a stabilizer. Both stabilized the oil droplets in the emulsion prior to spray drying. Matsuno and Imagi (1991) have shown that emulsion activity and emulsion stability influenced the amount of encapsulated oil. We hypothesize that the emulsion, stabilized by lecithin and Avicel, resulted in better encapsulation during spray drying and thus produced stable microcapsules. This suggested that emulsion characteristics before spray drying closely affected quality of microcapsules.

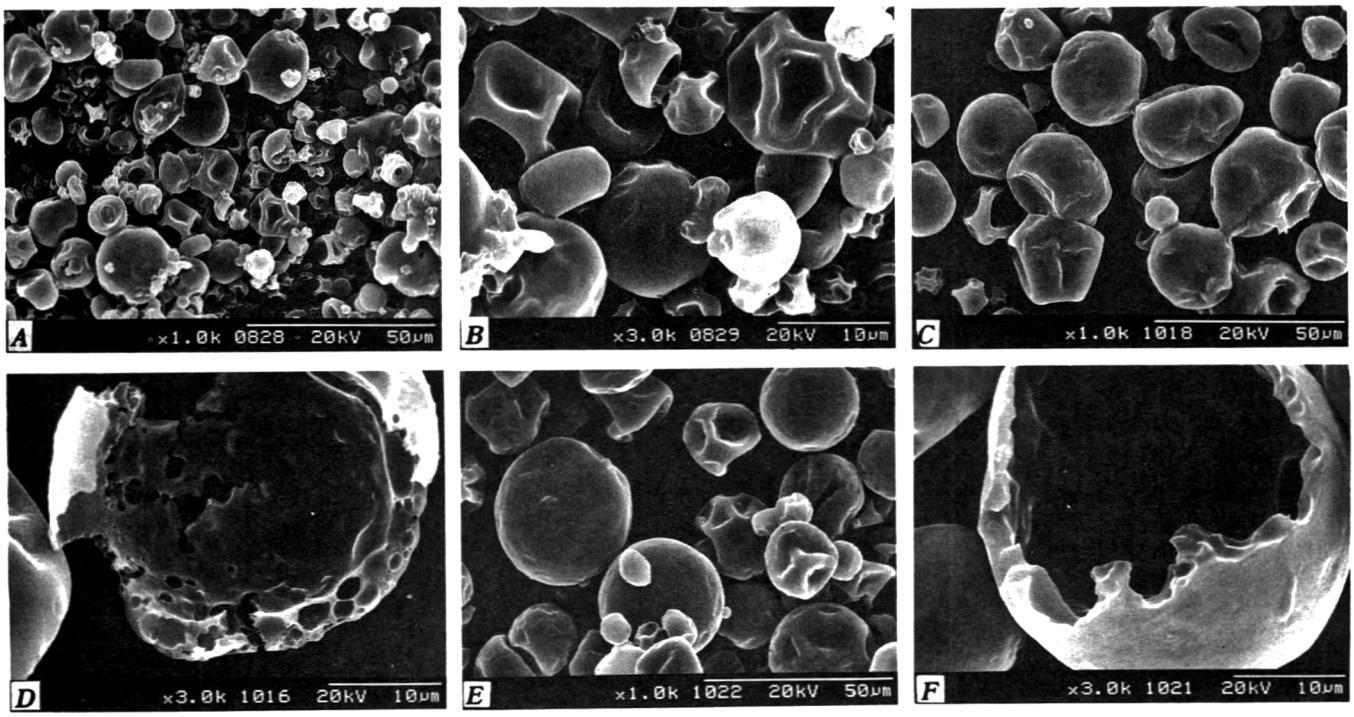


Fig. 1—Scanning electron micrographs of squid oil microcapsules of different formulations: (A,B)CM; (C,D)GCM; (E,F)GCML.

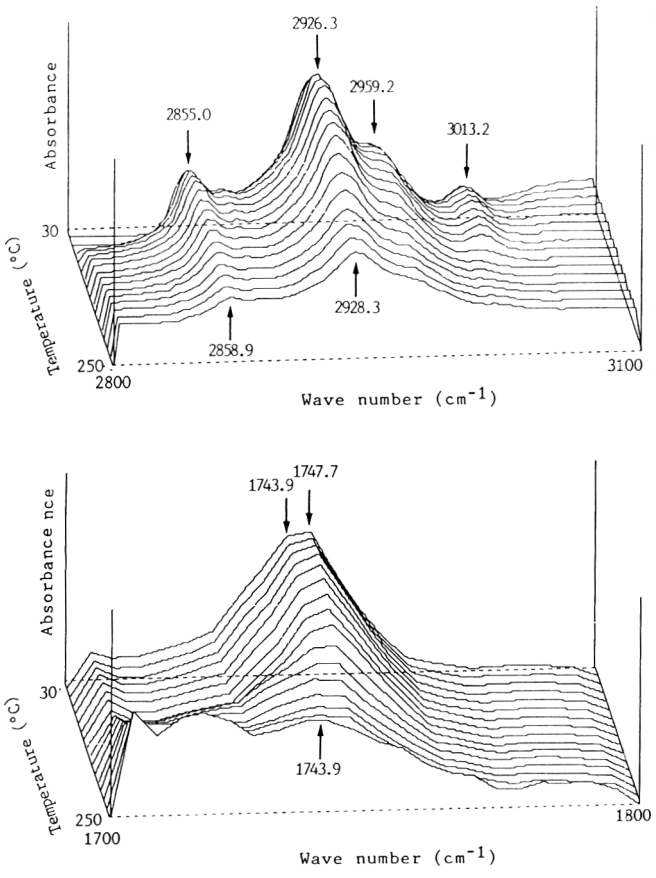


Fig. 2—IR spectra of squid oil microcapsule (GCM) as related to temperature.

Table 4—Temperature of DSC exothermic peak exhibited by squid oil microcapsules

Code	Temperature (°C)	
	Onset	Peak
Oil	135.72	153.15
Oxidized oil[a]	123.16	140.55
GCM	131.40	163.53
GCM20	136.47	158.94
GCML	145.72	185.14

[a] Oil was oxidized at 50°C in the dark for 4 days.

The surface topograph and inner structure of microcapsules prepared by different formulations were compared (Fig. 1). Obviously, extensive cracking appeared on the surface of the microcapsules CM. This might be the reason for the poor protection quality (Table 2) and the low value of the polyene ratio (Table 3). On the other hand, the topographs of both microcapsules GCM and GCML showed a smooth surface. There were no dents, pores or cracking. The outer appearance and particle-size of both microcapsules were similar [Fig. 1(C) and 1(E)], but wall-structures were different [Fig. 1(D) and 1(F)]. SEM clearly indicated that the wall layer of microcapsules GCML [Fig. 1(F)] had a tight, compact structure, but a microporous structure [Fig. 1(D)] was observed in the wall layer of microcapsules GCM. The emulsion stability before and during spray drying might influence the microcapsule wall structure. The emulsifier and stabilizer might decrease void-formation in microcapsules (Rosenberg et al., 1985). The compact structure of the wall could prevent squid oil from oxygen reaction.

The oxidative and thermal stabilities of edible fats and oils were successfully determined by PDSC and found to correlate well with the active oxygen method (Hassel, 1973). An oxidation onset temperature on the exotherm might be used to predict oxidative stability of squid oil with or without microencapsulation. The degradation onset temperatures and peak temperatures of PDSC thermograms for different samples were compared (Table 4). The oxidized oil and fresh oil clearly showed different degradation onset temperatures, 123.16°C and 135.72°C, respectively, indicating that the oxidized oil more readily degraded than fresh oil. Although squid oil was microencapsulated (GCM and GCM20), the degradation onset and peak temperatures were similar to the unencapsulated oil. This shows that microcapsules GCM and GCM20 did not improve stability, as indicated also in the results of the polyene ratio. On the other hand, microcapsules GCML exhibited considerably delayed degradation-onset and peak temperatures, suggesting better protection for squid soil microcapsules. The better wall-structure of microcapsules GCML might be responsible for the highest thermal stability.

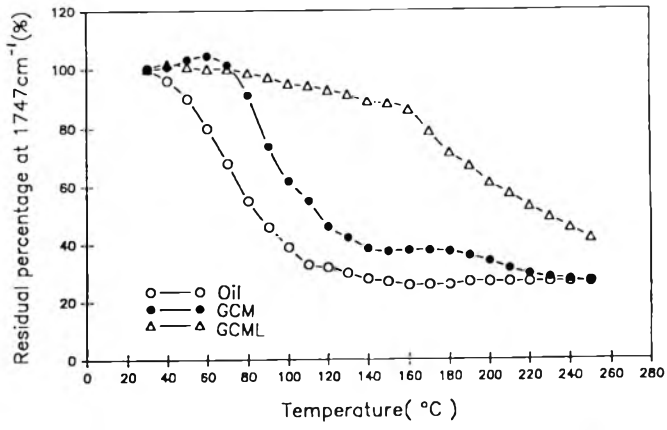


Fig. 3—Effect of temperature on absorbance at 1747 cm^{-1} of oil and microcapsule (GCM and GCML).

The microscopic structural changes associated with the thermal response of unencapsulated or encapsulated oils were investigated with a DSC-FTIR microscope spectrometer (Fig. 2). The absorbance at 2855 cm^{-1} and 2926 cm^{-1} were assigned to the symmetric and asymmetric stretching of CH_2 chains for fatty acids in oil, respectively. The peak at 1747cm^{-1} was assigned to the carbonyl group of triglycerides. Absorbances of those main peaks for fresh oil decreased considerably with increase of temperature. Similar results were obtained for squid oil microcapsules, but the decreasing tendency was slower than that of fresh oil. The temperature-dependent degradation of squid oil might be responsible for these results. Temperature courses of thermal degradation of unencapsulated oil and encapsulated oils GCM and GCML were compared (Fig. 3). Unencapsulated oil started to degrade at 30°C with almost no induction period, while the encapsulated oils showed an induction period and GCML was stable until about 160°C.

This indicated that the microencapsulation technique effectively protected squid oil from thermal degradation. Moreover, GCML showed effective thermal stability.

CONCLUSION

MICROENCAPSULATION could effectively enhance oxidative and thermal stabilities of squid oils. Inclusion of lecithin and Avicel in the wall material formulation containing macromolecules could further improve encapsulating effectiveness and oxidative and thermal stabilities. Both PDSC and DSC-FTIR could be used to evaluate stability of microcapsules and they correlated well with polyene ratio.

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Particle Size and Mixing Time Effects on Sensory and Physical Properties of Low-fat, High-moisture Pork Frankfurters

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ABSTRACT

Changes in particle size affected measured characteristics more than changes in mixing time. Sensory results indicated that use of a 2.0 mm plate produced the coarsest ($P<0.05$) product. CIE b* values decreased (<0.05) as particle size was increased. Products produced with a 2.0 mm plate compared to a 1.4 mm plate had higher ($P<0.05$) hardness values and total energy to shear. Particle size did not affect ($P>0.05$) cooking loss, springiness, cohesiveness, or chewiness. Additional mixing tended to decrease sensory particle size and increase springiness and chewiness.

Key Words: low-fat, pork, frankfurters, texture, physical properties

INTRODUCTION

LOW-FAT, HIGH ADDED WATER (AW), finely comminuted cured processed meats formulated to maximize the 40% sausage requirement (fat plus AW $\leq$ 40 and fat $\leq$ 30% in the finished product) usually result in products that are soft and have excessive purge during storage (Claus et al., 1989, 1990; Claus and Hunt, 1991). Increasing the sodium chloride content can result in firmer texture (Hand et al., 1987). However, excessive intake of sodium has been related to increased incidence of hypertension (Dahl, 1972). Numerous textural modifying adjuncts have been tested in low-fat processed meats. Incorporation of kappa- and iota-carrageenan into low-fat (11–12%), low added water (<10%) processed meats reduces moisture loss and, for kappa-carrageenan samples, increased hardness (Foegeding and Ramsey, 1986). Lin et al. (1988) reported that frankfurters containing carboxymethyl cellulose had lower hardness values than low-fat controls.

Mixing develops uniform distribution of fat and lean particles (Pearson and Tauber, 1984). In addition, extended mixing can reduce the visibility of particles (Gregg et al., 1993). Extended mixing of postminced batter (2°C) resulted in (numerically) higher hardness values in cooked frankfurters (Sylvia, 1992). Barbut (1988) reported that products formulated with mechanically deboned chicken meat and processed with a short chopping time were harder than those chopped longer. However, short chopping time products resulted in softer products when phosphates were incorporated. Barbut (1990) reported that a greater reduction of the lean particle size during comminution resulted in lower hardness values in pork bologna.

Our objectives were to determine the effects of increasing particle size (1.4, 1.7, 2.0 mm) of the lean fraction and postmince mixing time (5, 15 min) on the visibility of particles and physical properties of low-fat, high moisture pork frankfurters.

MATERIALS & METHODS

Product manufacture

Lean and fat raw materials were taken from hams and loins of four pork carcasses at the VPI&SU meats laboratory. All raw meat materials were frozen (−29°C) and stored (−29°C) for 5 mo in oxygen impermeable vacuum bags (Cryovac Division, W.R. Grace and Co., Duncan, SC) after sampling. The lean pork was cut with a band saw (model 5216, Hobart Manufacturing Co., Troy, OH) into cubes to facilitate grinding. The lean frozen cubes were ground through a 9.5 mm and 3.2 mm grinder plate with a meat grinder (model 4532, Hobart Manufacturing Co., Troy, OH) and mixed for 5 min (model #100-DA, Leland Meat Mixing Machine, Leland Detroit Mfg. Co., Detroit, MI) prior to sampling for chemical analysis.

Frozen fat (pork backfat) was also cut into cubes and ground using a 9.5 mm grinder plate prior to chopping in a bowl chopper (model 8142, Hobart Manufacturing Co., Troy, OH) for 5 min. Lean and fat samples were stored (4°C) in plastic lugs for 18 hr. Both lean and fat samples were analyzed for moisture (AOAC, 1990), fat (Fosslet Rapid Fat Analyzer, Foss Food Technology Corp., Eden Prairie, MN), and protein (estimated by difference) in order to formulate the frankfurters.

The products were formulated using the Least Cost Formulator™ (Least Cost Formulations Ltd. Inc., Virginia Beach, VA) to contain: 15% fat, 25% added water, 0.5% sodium tripolyphosphate (Stauffer Chemical Co., Shelton, CT), 2.3% sodium chloride, 2% sucrose, and 0.42% frankfurter seasonings (Heller Co., Bedford Park, IL) on a finished weight basis, and 156 ppm sodium nitrite and 0.044% sodium erythorbate on a meat block basis.

Of the six treatments, each had the same process with the exception of plate size and post-mince mixing time. Coarse ground lean pork was mixed 5 min with the salt, phosphate, nitrite, sucrose, and water in a bowl mixer (model A-200, Hobart Manufacturing Co., Troy, OH) using a dough-hook attachment. This mix was processed with a Mincemaster™ (model GL-86, Griffith Design and Equipment Co. Chicago, IL) using the appropriate particle size plate. After mincing, the batch (5.8 kg) was divided into halves and the remaining ingredients; erythorbate, frankfurter seasoning, and fat trim were mixed in a Kitchenaid® mixer (model K45SS, Hobart Inc., Troy, OH) for 5 or 15 min. This mix was again minced through the appropriate size plate. To minimize air pockets the batter was placed on a plastric tray at a depth of $\approx$ 2 cm and evacuated twice at 30% and once at 100% of full capacity using a vacuum packager (model VC 999-01, VC999 Inauen Packaging Equipment Corp., Gladstone, MO). The batter was stuffed (model E251, Koch Piston Stuffer, Koch Supplies, Inc., Kansas City, MO) into 34 mm cellulose casings (Teepack Co., Chicago, IL). After stuffing, the links ($\approx$ 25 cm, length) were made by hand and heat-processed in a smokehouse (Alkar Engineering Corp., Lodi, WI) to internal temperature 71°C determined with a hand-held thermometer. The smokehouse schedule was: 20 min at dry bulb 125°C and wet bulb 0°C, 15 min at dry bulb 150°C and wet bulb 132°C with smoke, 15 min at dry bulb 170°C and wet bulb 150°C with smoke, steam-cooked until internal temperature was achieved (about 4 min) and showered 5 min. After cooking the frankfurters were stored in covered plastic lugs at 3°C for 15 hr.

Cooking losses

All of the frankfurters were used in determining cooking losses and were weighed prior to heat processing. After the cooked frankfurters were chilled (4°C) for 15 hr, the products were reweighed. Cooking loss was expressed as a percentage difference between the post processing and chilled weights.

Texture measurements

Texture profile analysis procedures reported by Bourne (1978) and Montejano et al. (1985) were used. Texture analyses were performed on chilled (4°C) samples stored horizontally in plastic lugs for 5 days. Six to 8 cores per treatment were tested and averaged for each reported characteristic. A core of the frank (19 mm diameter by 19 mm height) was axially compressed to a height of 4.75 mm (75% compression) to determine total energy and hardness. To determine springiness, cohesiveness, and chewiness, a core was compressed twice to 50% of its height. An Instron (model 1011, Instron Corp., Canton, OH) was programmed for a load range of 5 kg (10% of 50 kg compression load cell) with a crosshead speed of 100 mm/min. The chart recorder was set for 200 mm/min chart speed.

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Sensory evaluations

A seven-member sensory panel was used to evaluate visual particle size in the cooked frankfurters. The general guidelines for training the panel were as reported by Civille and Szczesniak (1973). Frankfurters were vacuum-packaged and stored for 21 days (4°C) prior to evaluation. Panelists were asked to evaluate 10 cm long axially sliced links displayed at 4°C under 1076 lux (fluorescent light). Scores for particle size were assigned based on an 8-point scale (1 = extremely coarse to 8 extremely fine). Adverbs used for particle size were extremely (1 and 8), very (2 and 7), moderately (3 and 6), and slightly (4 and 5).

Chemical and color measurements

All treatments (cooked product) were analyzed for moisture, fat, and protein following AOAC (1990) procedures. Frankfurter samples were ground, mixed, and packaged in oxygen impermeable bags (Cryovac Division, W.R. Grace and Co., Duncan, SC). Samples were blast-frozen and stored (−40°C) for 3 mo prior to chemical analysis.

A chroma meter (model CR-200, Minolta Camera Co., Ltd., Osaka, Japan) with 8 mm diameter aperture, referenced vs a white calibration plate (L* 97.91, a* −0.74, b* 2.45), was used to measure the CIE L*, a*, b* values. Frankfurters used for color measurements were stored (4°C) for 5 days and then sliced perpendicular to the length at equal increments (4.75 mm). Six to 8 cores were measured per treatment.

Statistical analysis

Data (three replications) were analyzed by the ANOVA procedure of SAS Institute, Inc. (1990) as a 3×2 factorial, split plot design with particle plate size representing the whole plot and post-mince mixing time as the split plot. When significant model effects were determined in independent variables, treatment means were separated using the Least Significant Difference procedure of SAS Institute, Inc. (1990).

RESULTS & DISCUSSION

Chemical analysis and cooking loss

Products were formulated for 15% fat, 25% added water based on cooking loss of 6%. The chemical analysis indicated that fat content (SD = 1.01) and added water (moisture, SD = 1.62; protein, SD = 0.31) were 1.3% and 2.7% lower than the targeted formula, respectively.

Cooking losses were not (P>0.05) affected by particle size or mixing time (Tables 1 and 2). Gregg et al. (1993) found that massaging up to 2.5 hr did not affect cooking losses. Foegeding and Ramsey (1986) suggested that evaporation rate and fat-to-protein ratio were the main regulators of moisture losses during cooking.

Sensory analysis

In general, sensory particle size values decreased (coarser) as particle plate size increased (Table 1). Frankfurters produced using the 2.0 mm plate had the coarsest (P<0.05) particles. Shorter chopping times result in larger fat globules (Barbut, 1988, 1990). No visual difference occurred in particle size between the 1.4 mm and 1.7 mm plates.

Mixing time did not affect (P>0.05) visual particle size. In contrast, Gregg et al. (1993) reported that extended mixing (2.5 hr) reduced the perception of particles.

Color analysis

No differences (P>0.05) occurred in the CIE L* and a* due to particle size (Table 1). However, as particle size decreased, CIE b* value increased (P<0.05). CIE L*, a*, and b* values were not affected (P>0.05) by mixing time (Table 2). Gregg et al. (1993) also found no differences in lightness or off color with increases in massaging time.

Texture analysis

In general as particle size increased, the total energy increased (Table 3). Particle size of 2.0 mm resulted in higher (P<0.05) total energy than particle size of 1.4 mm. However, neither size produced frankfurters different in total energy from the 1.7 mm particle size. Particle sizes of 1.7 mm and 2.0 mm frankfurters were not different (P>0.05) in hardness, but were firmer (P<0.05) than the 1.4 mm produced frankfurters. Barbut (1988, 1990) indicated that greater reduction of lean meat particle size resulted in lower hardness values. *—Continued on page 54*

Table 1—Relationship of particle plate size to some chemical, physical, and sensory properties of low-fat pork frankfurters

Property	Particle plate size (mm) 1.4	1.7	2.0	Standard error
Chemical analysis (%)				
Moisture	68.5	68.7	70.1	0.44
Fat	13.7	13.6	13.8	0.51
Protein	11.7	11.9	11.6	0.14
Losses (%)				
Cooking	7.4	7.2	7.6	0.17
CIE values				
L*	70.2	69.6	68.8	0.36
a*	12.2	12.1	12.2	0.24
b*	8.0[a]	7.6[b]	7.2[c]	0.08
Sensory[d]				
Particle size	7.2[a]	6.8[a]	5.8[b]	0.17

[abc] Means within same row with unlike superscripts are different (P<0.05). Means within same row without superscripts are not different (P>0.05).

[d] Sensory particle size: Based on scale 1 to 8: 1 = extremely coarse; 8 = extremely fine.

Table 2—Relationship of mixing time to some chemical, physical, and sensory properties of low-fat pork frankfurters[a]

Property	Mixing Time (min) 5	15	Standard error
Chemical analysis (%)			
Moisture	69.3	68.9	0.67
Fat	13.4	14.0	0.34
Protein	11.7	11.8	0.10
Losses (%)			
Cooking	7.7	7.1	0.17
CIE values			
L*	69.6	69.6	0.14
a*	12.0	12.3	0.14
b*	7.6	7.7	0.07
Sensory[d]			
Particle Size	6.3	6.8	0.19
75% Instron compression			
Total energy (kg × mm)	15.9	16.6	0.27
Hardness (kg)	2.3	2.4	0.05
50% Instron compression[e]			
Peak force (kg)	1.7	1.8	0.04
Springiness (b2)	12.0[b]	12.6[c]	0.17
Cohesiveness (TE_2/TE_1 × 100)	27.5	28.0	0.31
Chewiness (kg × mm)	7.2[b]	8.1[c]	0.29

[a] Means within same row without superscripts not different (P>0.05).

[bc] Means for springiness and chewiness with unlike superscripts are different (P<0.057).

[d] Sensory particle size: Based on scale 1 to 8: 1 = extremely coarse; 8 = extremely fine.

[e] 50% compression: Springiness = b2, base width (mm) of second compression; cohesiveness = TE_2/TE_1 × 100, total energy of first compression over the second; chewiness = hardness (50%, peak force) × springiness × cohesiveness/100.

Table 3—Relationship of particle plate size to textural properties of low-fat pork frankfurters

Property	Particle plate size (mm) 1.4	1.7	2.0	Standard error
75% Instron compression				
Total energy (kg × mm)	15.0[a]	16.3[ab]	17.4[b]	0.46
Hardness (kg)	2.2[a]	2.4[b]	2.5[b]	0.06
50% Instron compression[c]				
Springiness (b2)	12.4[a]	12.6[a]	12.0[a]	0.21
Cohesiveness (TE_2/TE_1 × 100)	27.6[a]	28.9[a]	26.7[a]	1.04
Chewiness (kg × mm)	7.2[a]	8.5[a]	7.2[a]	0.38

[ab] Means within same row with unlike superscripts are different (P<0.05), except for total energy (P<0.056).

[c] 50% compression: Springiness = b2, base width (mm) of second compression; cohesiveness = TE_2/TE_1 × 100, total energy of first compression over the second; chewiness = hardness (50%, peak force) × springiness × cohesiveness/100. Peak force (50%) data not shown.

Sensory Characteristics of Frankfurters as Affected by Fat, Salt, and pH

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ABSTRACT

Acceptable processing and flavor profile characteristics of frankfurters were achieved near a pH of 6.0. As salt increased, hardness, juiciness, saltiness, and flavor intensity scores increased. As fat increased, juiciness scores decreased (at salt levels > 1.3%) due to substitution of water for fat. In addition, increased fat resulted in decreased off-flavor. Model predictions suggested that acceptable frankfurters could be manufactured with a minimum of 11.25% fat and 1.3% salt at pH 6.0.

Key Words: frankfurters, beef, fat, salt, acidity

INTRODUCTION

OVER 2.18 BILLION KG OF SAUSAGE products are produced in the U.S. annually, including over 60 million kg of frankfurters and 282 million kg of bologna (AMI, 1989). These emulsified sausage products contain up to 30% fat and > 2% salt. To make such products more acceptable to health-conscious consumers, the fat and salt content should be reduced while minimizing changes in product palatability. Dietary guidelines for Americans (USDA and USDHHS, 1985) suggested reducing sodium, fat, and cholesterol in diets. The American Health Association suggested limiting fat consumption to < 30% of calories, to consume < 300 mg cholesterol, and <3g sodium/day (NRC, 1989). Fresh cuts of beef and pork have low sodium ≈ 70 mg/100g, cooked basis); however, one frankfurter can contribute more than 14 times that amount of sodium (Maurer, 1983).

Commercially available emulsion products typically contain 30% fat and 12% protein. Keeton (1983) investigated effects of varying fat levels (from 20% to 30%) on physical and sensory characteristics of pork patties. Juiciness was the only sensory attribute to differ and reduced compression energy for the 30% fat patties was the only change observed using texture profile analysis.

The amount of sodium chloride that can be replaced in meat products is limited (Hand et al., 1982). However, it may be reduced, provided changes in physical and sensory properties of the products are minimized. Trout and Schmidt (1987) reported that as salt content of an emulsified product decreased from 2.93% to 2.13% and 1.33%, a decrease occurred in temperature to which the product could be heated before water-binding ability was affected (from 86°C to 63°C to 57°C, respectively). Whiting (1984) reported that raw emulsions made with reduced salt levels (below 2.0%) had a sharp increase in water released by the batter. Gel strength gradually decreased with salt reductions from 3.5% to 1.0%, and fat was released in emulsions containing < 1.0% salt. Seman et al. (1980) evaluated physical properties of bologna made with 2.5% salt vs a similar product made with 1.25% salt. The lower ionic strength product showed increases in fat released, gel liquid released, and total cookout in emulsion stability tests. Instrumental evaluation of textural characteristics indicated that salt reduction resulted in lower hardness, compressed peak force, gumminess scores, and increased elasticity.

Poulanne and Terrell (1983a,b), investigated effects of salt preblending prior to processing. They reported that salt reduction from 2.5% to 1.5% resulted in minimal physical and chemical changes, but the products tasted less "salty." Salt reduction to 1.0% resulted in decreased water-binding capacity despite preblending treatments.

Production of reduced fat and salt frankfurters should have widespread appeal to health-conscious consumers. However, fat and salt contribute to many sensory properties characteristic of frankfurters. According to USDA (1988) guidelines, water may replace fat in the formulation on an equal weight basis. Increased water may affect texture and juiciness. Reducing salt content decreases protein extraction and water binding in addition to altering palatability attributes. Some water-binding properties that could be lost by reducing salt content may be partially compensated by altering pH of the raw meat batter.

Matulis et al. (1994) reported that commercial frankfurters had a wide range of textural and sensory characteristics. Inclusion of the mean ± one standard deviation for each characteristic included about 66% of the products assessed. Because those products have been commercially successful, they presumed they were acceptable.

Our objective was to evaluate effects of fat, salt, and pH on sensory characteristics of frankfurters. From such information, the ranges of fat and salt levels that could produce acceptable frankfurters could be delineated.

MATERIALS & METHODS

Experimental design

Continuous variables of salt, fat, and pH, were evaluated simultaneously to formulate low salt/low fat frankfurters with acceptable palatability attributes. A central composite design (CCD) (Table 1) was used to evaluate results using response surface methods (Box et al., 1978). The CCD consists of a 2^k factorial design, 2k star points, and several center points. The star points of a rotatable CCD for k = 3 are ± 1.732($\sqrt{3}$). The star points (± 1.67 for fat and salt, ± 1.5 for pH) were chosen based on the range of values within practical constraints of the experiment. The target fat, salt, and pH values are shown in Table 1. The amounts of fat and salt used are shown in Table 2.

Eight batches of frankfurters were manufactured initially in a 2 × 2 × 2 factorial design (pH = 5 or 7; salt = 1.0% or 2.5%; and fat = 10% or 25%). Following initial analyses, the remaining nine batches of frankfurters were manufactured to complete the CCD.

Frankfurter production

Two masterblocks of lean beef trim (5% fat) and beef fat (90% fat) were used to formulate frankfurters to the desired fat level. Lean trim was obtained from boneless rounds trimmed of subcutaneous and intermuscular fat. Beef fat (subcutaneous and intermuscular deposits) was obtained from USDA Choice wholesale beef cuts. Raw materials were ground through a 2.5-cm plate, mixed thoroughly, vacuum-packaged (B540 bags, CryoVac, W.R. Grace, Simpsonville, SC), evacuated at 1 kp/cm², and frozen (−30°C) for subsequent use. Lean and fat trim were allowed to temper (4°C) 48 hr prior to formulation and processing.

All frankfurters were formulated to result in a final product with 40% added water plus fat in compliance with USDA standards for frankfurter production (USDA, 1988). Additional water was included in the for-

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Table 1—Experimental design for response surface model for characteristics of frankfurters with varied pH, fat, and salt

Run	X_1 (% fat)	X_2 (% salt)	X_3 (pH)
1	+1	+1	+1
2	+1	−1	+1
3	−1	+1	+1
4	−1	−1	+1
5	+1	+1	−1
6	+1	−1	−1
7	−1	+1	−1
8	−1	−1	−1
9	0	0	0
10	0	0	0
11	0	0	0
12	0	0	−1.5
13	0	0	+1.5
14	0	−1.67	0
15	0	+1.67	0
16	−1.67	0	0
17	+1.67	0	0

	Coded matrix variables						
Treatment	−1.67	−1.5	−1	0	+1	+1.5	+1.67
X_1 (% fat)	5.0	—	10.0	17.5	25.0	—	30.0
X_2 (% salt)	0.5	—	1.0	1.75	2.5	—	3.0
X_3 (pH)	—	4.5	5.0	6.0	7.0	7.5	—

mulation to compensate for evaporative loss during the cooking cycle. When an additional ingredient such as sodium bicarbonate was added, or when the amount of sodium chloride was varied from 2.5%, a proportional amount of water was removed from the formulation. Concentrations of all other components were unaffected. Frankfurter formulations are presented in Table 2.

Production order was determined by pH of the product. All frankfurters formulated to the same pH value were manufactured at the same time to avoid cross-contamination. Equipment was thoroughly rinsed between pH changes to minimize cross-contamination. Within pH groups, frankfurters were produced in order from low to high salt concentration.

Lean beef was chopped for 1 min in a Kramer-Grebe Silent Cutter (T. W. Kutter, Inc., Avon, MS) along with salt, Griffith's Prague Powder (6.25% sodium nitrite), and sodium erythorbate. Fat, spice mix, and half the water were added to the bowl cutter, then product was chopped an additional 2 min. Nonfat dry milk, pH altering ingredients, and remaining water were added, and batter was chopped an additional 1.5 min to ≈ 13°C. Batter was transferred to a Bench Mince Master emulsifier (model GL 86, Griffith Design and Equipment Co., Chicago, IL) and processed using a 2.5-mm plate.

Batter was transferred to a Zuber stuffer and stuffed into 25-mm diameter cellophane casings. The product was hand-linked into 13-cm lengths and tagged for identification. Frankfurters were weighed, then cooked in a Vortron single cart smokehouse (Vortron Co., Beloit, WI) using the following schedule: 20 min, dry bulb (DB) = 54.4°C, smoke off; 20 min, DB = 65.6°C, wet bulb (WB) = 57.2°C, smoke on; 20 min, DB = 71.1°C, WB = 62.8°C, smoke on; to an internal temperature of 68°C in five randomly located frankfurters, DB = 76.7°C, WB = 71.1°C, smoke on. Frankfurters were removed from the smokehouse, weighed for cooking yield, cold-showered for 10 min, then held at 4°C overnight. About 15 hr after chilling, casings were removed, frankfurters were vacuum-packaged (CryoVac type B540 bags, W. R. Grace, Simpsonville, SC, evacuated at 1 kp/cm², Multivac type AGW, Koch, West Germany) into ≈ 0.5-kg packages, then stored (4°C) for subsequent evaluation.

Chemical analysis

Proximate analysis for fat and moisture was performed in triplicate within 48 hr as described by Riss et al. (1983). Ultimate pH was evaluated 24 hr after manufacturing as described by Koniecko (1985). Frankfurters were re-vacuum-packaged following sample removal for each analysis.

Sensory evaluation

Sensory evaluation of frankfurters was conducted using a 6-member panel trained as described by Civille and Szczesniak (1973). Panelists were trained during eight 1-hr sessions; initial sessions were conducted to familiarize panelists with sensory characteristics to be evaluated. Definitions and anchors were drawn from the work of Munoz (1986), Szczesniak (1963), and Szczesniak et al. (1963, 1975), and included: hardness: force required to bite completely through sample between molar teeth; cohesiveness: degree to which mass holds together after mastication; juiciness: amount of moisture perceived during mastication; saltiness: amount of saltiness perceived during mastication; flavor intensity: amount of pronounced flavor characteristic of the product; off-flavor intensity: amount of flavor not normally present in the product (may often be considered offensive).

Frankfurters were manufactured to represent the range of each sensory characteristic to serve as guidelines for evaluation. One batch of standard frankfurters was manufactured as previously described to represent the center point for all sensory characteristics and was described as having no off-flavor. The standard frankfurters were manufactured to contain 17.5% fat and 1.75% salt at pH 6.0. These frankfurters were presented to panelists during each session to serve as reference. Panelists scored each sample using a 15-cm unstructured line scale.

Frankfurters were steeped in an 82°C waterbath to internal 65°C (5 min). Two 1.5-cm samples of each frankfurter were served to each panelist. Panelists rinsed between samples, first with warm apple juice to relieve salty sensation, then with water to return to a neutral palate. All frankfurter treatments were evaluated for sensory characteristics in duplicate under red lights in sensory booths. Panelists received 6 samples/session in random order.

Statistical analysis

Bartlett's test for homogeneity of variance (Bartlett, 1947) was conducted to determine if variance was the same for all six panelists × 17 tests (102 panelist/test combinations [two trials/combination/panelist]). If the hypothesis of a constant variance was rejected, then the replicate measurements that differed by more than 7.5 cm out of 15 cm were discarded. By sensory characteristic, the number of data points discarded was hardness-4, cohesiveness-4, juiciness-3, saltiness-2, flavor intensity-2, and off-flavor intensity-5.

All equations and response surface plots were generated using a Response Surface Methodology program (Anonymous, 1989). The response surface was characterized using the model shown in Table 3.

RESULTS & DISCUSSION

THE INFLUENCES OF SALT, FAT, AND pH on sensory characteristics of frankfurters were expressed as contour plots. These were two-dimensional plots with two continuous variables on the X and Y axes, while the third was held constant. The test for homogeneity of variance (Table 4) indicated that panelists tended to be inconsistent when evaluating sample replicates for all responses except saltiness and flavor intensity. The inconsistency of some attributes may indicate that some panelists needed more training. Regression equations developed to predict sensory characteristics from fat and salt content and pH are presented in Table 3.

The effect of salt and pH on sensory hardness with fat constant at 15% were compared (Fig. 1). Maximum hardness was achieved at ≈ pH 6.0 for all salt levels. As salt concentration increased, frankfurter hardness increased. These results agreed with those of Seman et al. (1980), who reported that salt reduction (from 2.5% to 1.25%) resulted in decreased hardness and Instron peak force in bologna. The salt concentration to achieve hardness scores of ≈ 6 (previously determined acceptable, Matulis et al., 1994) may be minimized at pH values between 5.8 and 6.3. As pH is reduced and approaches the isoelectric point of muscle proteins (pH 5.0), protein dissociation prior to matrix formation is minimized. If pH were reduced below the isoelectric point, more protein would be extracted producing a firmer frankfurter. However, at very low pH values some proteins may be denatured, becoming ineffective in emulsion formation (Hamm, 1986).

Hardness decreased as pH increased above 6.3. At higher pH, more water is bound and retained. Such increased moisture levels may result in softer texture. Hamm (1986) suggested that as beef muscle homogenate pH increased from the isoelectric point (≈ 5.0) to 10.0, water-holding capacity increased; as pH increased, repulsion of protein chains increased enabling them to attract water molecules.

Table 2—Frankfurter formulations

Ingredient (g)	Order of formulation												
	1	2	3	4	5	6	7	8	9	10	11	12	13
Lean[a]	2974	3042	3001	3085	2906	2974	2937	3025	2928	3001	2856	3060	2928
Fat[b]	1362	1362	445	445	1362	1362	445	445	994	990	999	117	994
Water	1207	1207	2093	2093	1207	1207	2093	2093	1789	1789	1789	2474	1789
Salt	137.5	55.0	137.5	55.0	137.5	55.0	137.5	55.0	96.3	27.5	165.0	96.3	96.3
Spice mix[c]	27.5	27.5	27.5	27.5	27.5	27.5	27.5	27.5	27.5	27.5	27.5	27.5	27.5
Prague pwdr.[d]	13.7	13.7	13.7	13.7	13.7	13.7	13.7	13.7	13.7	13.7	13.7	13.7	13.7
Erythorbate[e]	3.0	3.0	3.0	3.0	3.0	3.0	3.0	3.0	3.0	3.0	3.0	3.0	3.0
NFDM[f]	198.0	198.0	198.0	198.0	198.0	198.0	198.0	198.0	198.0	198.0	198.0	198.0	198.0
Lactic acid	—	—	—	—	82.5	82.5	82.5	82.5	—	—	—	—	—
Sodium bicarb.	24.0	24.0	24.0	24.0	—	—	—	—	—	—	—	—	—

[a] Lean beef trim (5% fat).
[b] Beef fat (90% fat).
[c] Griffith's Wiener Seasoning #011-8424, Griffith's Laboratories, Alsip, IL).
[d] Griffith's Prague Powder (6.25% sodium nitrite), Griffith's Laboratories, Alsip, IL).
[e] Sodium erythorbate.
[f] Nonfat dry milk.

Table 3—Regression coefficients to predict the sensory characteristics of low fat/low salt frankfurters[a]

Sensory characteristic	b_0[b]	b_1	b_2	b_3	b_{12}	b_{13}	b_{23}	b_{11}	b_{22}	b_{33}
Hardness	8.59*	+0.78*	+1.99*	+0.80*	−0.25	+0.94*	−0.20	−0.14	−1.01*	−2.18*
Cohesiveness	7.50*	−0.01	+0.36	+1.00*	+0.26	+0.27	−0.98*	+0.22	+0.30	−0.06
Juiciness	7.18*	−0.37	+0.91*	+1.49*	−0.82*	−1.38*	+1.09*	−1.34*	+0.03	+0.51
Saltiness	6.67*	+0.45	+2.55*	+0.05	−0.24	−0.15	−0.43	0.00	−0.11	−1.31*
Flavor intensity	7.18*	+0.51*	+2.29*	+0.53*	−0.24	−0.37	−0.40	+0.05	−0.48*	−1.58*
Off-flavor intensity	14.31*	+1.65*	−0.35	+1.28*	+0.41	−0.07	+0.64	−0.85*	+0.69*	−3.69*

[a] Regression coefficients to fit the equation $y = B_0 + B_1X_1 + B_2X_2 + B_3X_3 + B_{12}X_1X_2 + B_{13}X_1X_3 + B_{23}X_2X_3 + B_{11}X_1^2 + B_{22}X_2^2 + B_{33}X_3^2 +$ error, where X_1 = % fat, X_2 = % salt, and X_3 = pH.
[b] b_0 is the least squares estimate of B_0.
* Significant at $P<0.05$.

Table 4—Bartlett's test for variance homogeneity for sensory characteristics of frankfurters

Characteristic	Calc chi-square value[a]	Hypothesis rejected[b]
Hardness	162.22	Yes
Cohesiveness	167.00	Yes
Juiciness	149.24	Yes
Saltiness	130.75	No
Flavor intensity	126.50	No
Off-flavor intensity	240.98	Yes

[a] Calculated chi-square value using Bartlett's test.
[b] If the calculated chi-square value is greater than chi-square (101, 0.99) = 136, then the assumption of a constant variance is rejected.

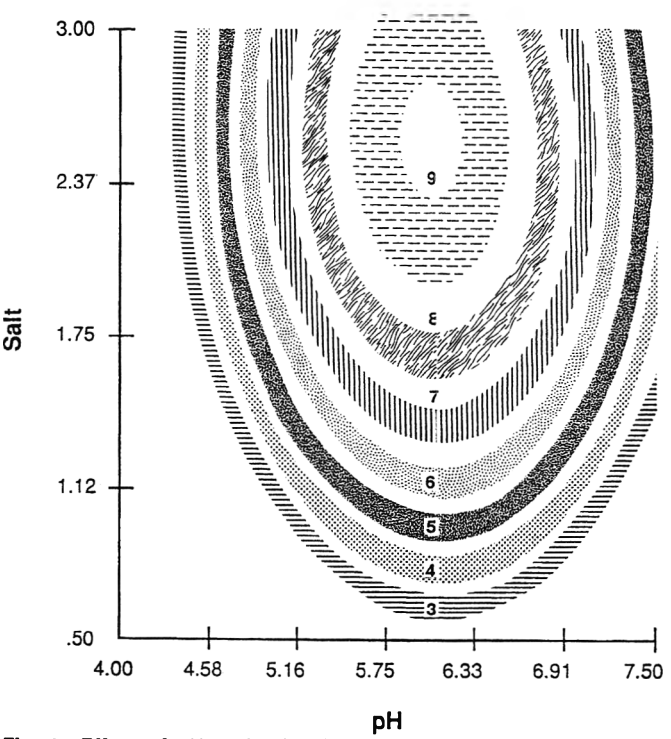


Fig. 1—Effect of pH and salt (%) levels on sensory hardness at 15% fat level. Response values are 0 = extremely soft (cream cheese); 15 = extremely hard (hard candy)

When salt concentration was constant at 2.0%, the firmest frankfurters were produced at ≈ pH 6.0 (Fig. 2). The salt-pH relationship was similar to that for hardness (Fig. 1). From pH values ≈ 5.2 through 6.9, hardness increased as fat content increased. This may be due to substitution of water for fat. Increased water decreased the force required to bite through the frankfurter. Claus et al. (1989) found that bologna firmness decreased as added water increased. They suggested that as added water increased, more hydrophilic regions of proteins associated with water instead of forming intermolecular protein-to-protein cross bridges.

Fat content had limited effects on sensory hardness at pH < 5.2. At pH near the isoelectric point, skeletal proteins (especially myosin) have few charged groups free to interact in network formation to trap water and fat in the batter. With reduced protein-protein interactions for matrix formation, frankfurters become softer.

At pH ≈ 6.0, relatively high hardness was achieved in reduced fat frankfurters. Altering the pH from this point would require addition of acidic or basic buffers that could cause flavor changes. The remaining experiments were conducted holding pH constant at 6.0 to avoid addition of such flavor-modifying components.

When pH was constant at 6.0, low salt concentrations (0.5% to 1.5% salt) had a greater emulsifying and stabilizing influence on hardness than did changes in fat concentration (Fig. 3). Salt assists in extraction of myofibrillar proteins that emulsify and stabilize batter. Poulanne and Terrell (1983a,b) found that salt levels could be reduced to 1.5% with minimal changes in physical properties of frankfurters. However, when salt was reduced to 1.0%, water-binding capacity was adversely affected.

At salt concentrations > 1.5%, modification in fat and salt content had similar effects on sensory hardness. Results suggested that a minimal amount of salt (≈ 1.5%) is required to

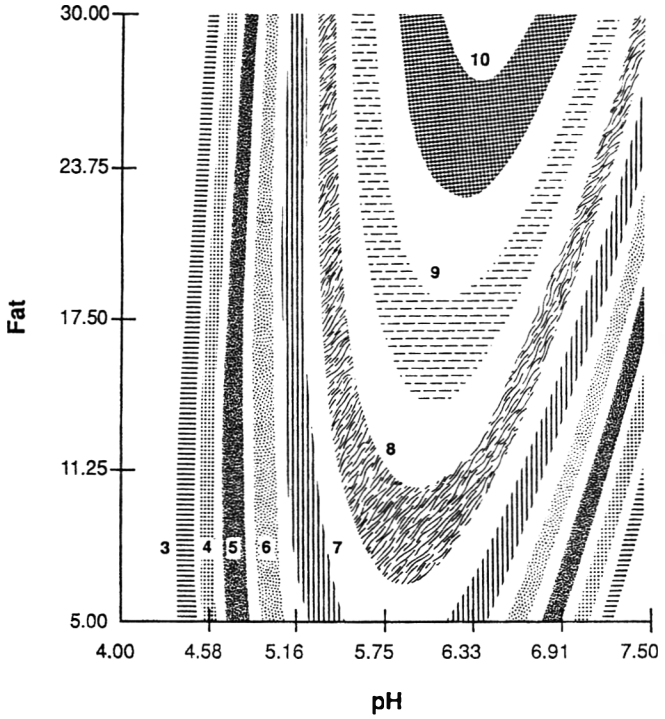


Fig. 2—Effect of pH and fat concentration on sensory hardness at 2% salt level. Response values are 0 = extremely soft (cream cheese); 15 = extremely hard (hard candy).

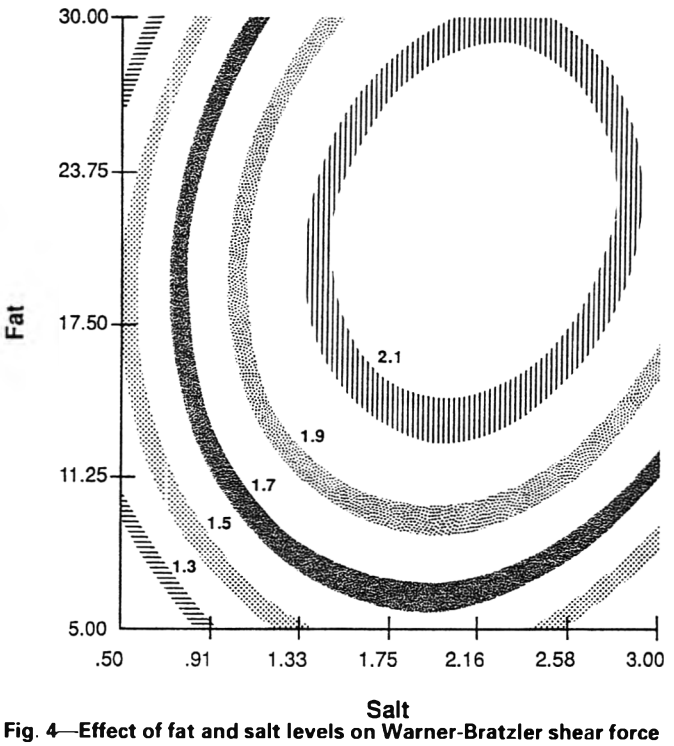


Fig. 4—Effect of fat and salt levels on Warner-Bratzler shear force (kg) values at pH 6.0.

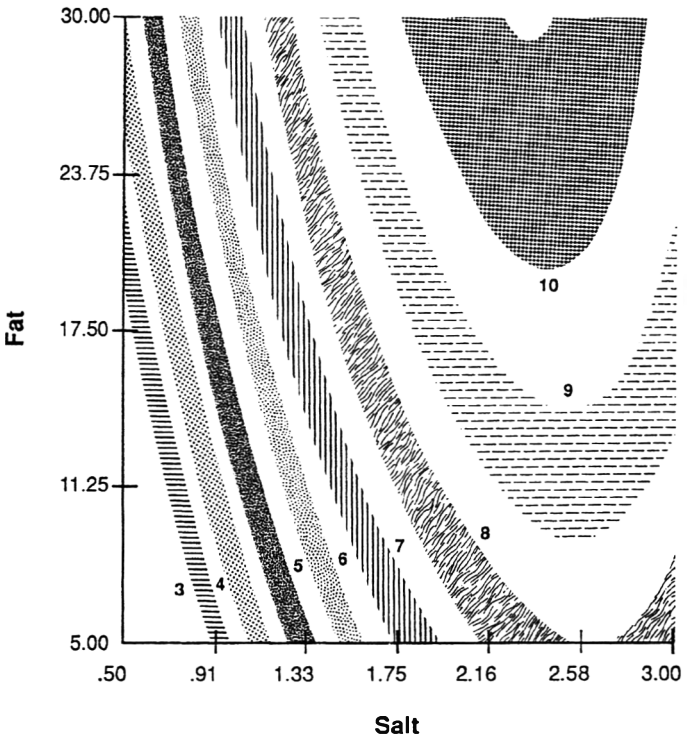


Fig. 3—Effect of fat and salt levels on sensory hardness at pH 6.0. Response values are 0 = extremely soft (cream cheese); 15 = extremely hard (hard candy).

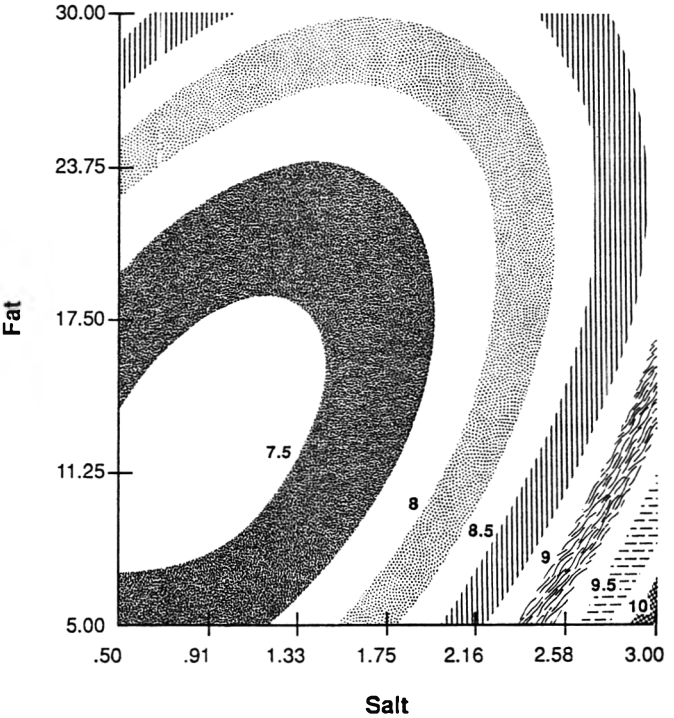


Fig. 5—Effect of fat and salt levels on cohesiveness at pH 6.0. Response values are 0 = extremely crumbly (carrot); 15 = extremely cohesive (biscuit dough).

extract protein and form a stable matrix. Trout and Schmidt (1987), studying the role of ionic strength on beef muscle homogenates, found that tensile strength of the homogenate was greatest between 1.29% and 1.87% salt.

The effects of salt and fat on Warner-Bratzler shear forces were similar to those for sensory hardness (Fig. 4). Increased fat and salt concentrations increased shear force values. Differences between shear force and sensory hardness may be attributed to differences between instrumental and sensory evaluation. No significant correlation was found between sensory hardness and shear force (Instron) of cold commercial frankfurters (Matulis et al., 1994).

The effects of fat and salt content on sensory cohesiveness were compared (Fig. 5). Cohesiveness was the least consistent sensory attribute among panelists. The panelists were trained to evaluate cohesiveness in frankfurters. However, the range of cohesiveness of these frankfurters may have been too low for panelists to make consistent judgments. As salt increased, frankfurter cohesiveness increased. This may be due to increased pro-

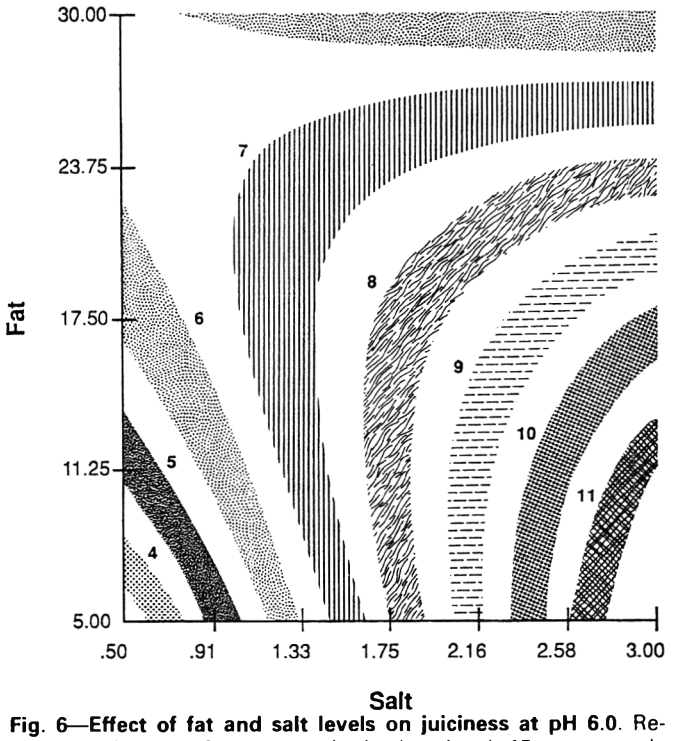


Fig. 6—Effect of fat and salt levels on juiciness at pH 6.0. Response values are 0 = extremely dry (crackers); 15 = extremely juicy (ripe tomatoes).

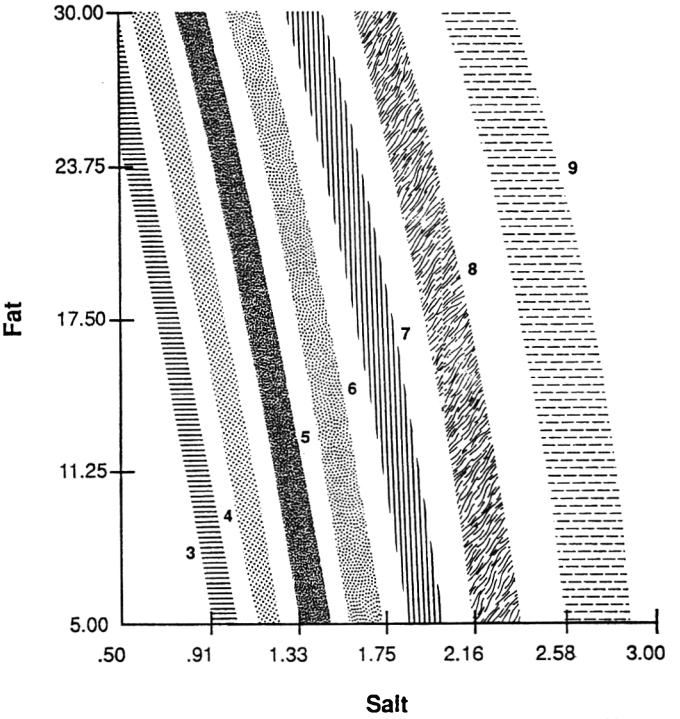


Fig. 8—Effect of fat and salt levels on flavor intensity at pH 6.0. Response values are 0 = extremely bland (tofu); 15 = extremely intense (pepperoni).

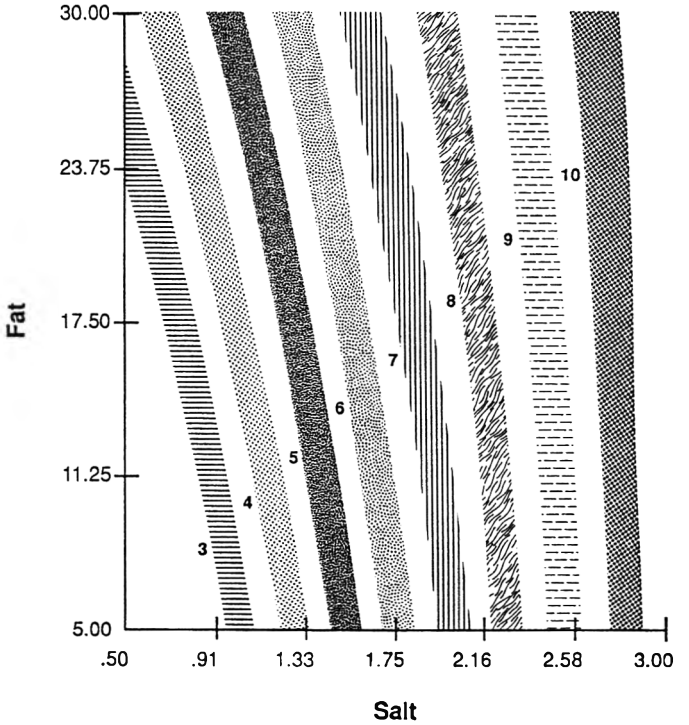


Fig. 7—Effect of fat and salt levels on saltiness at pH 6.0. Response values are 0 = extremely bland (water); 15 = extremely salty (pretzels).

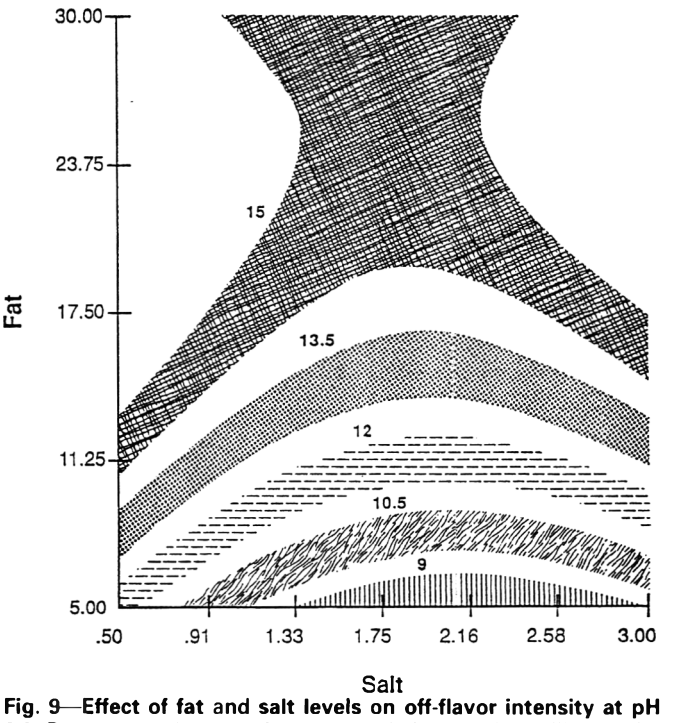


Fig. 9—Effect of fat and salt levels on off-flavor intensity at pH 6.0. Response values are 0 = extremely intense (metallic, rancid, fishy); 15 = extremely bland.

tein extraction resulting in more protein-protein interaction at higher salt levels. The most cohesive frankfurters resulted from reducing fat and increasing salt.

As salt concentration increased, juiciness increased (Fig. 6). Sodium chloride extracts myofibrillar proteins allowing formation of a water-binding protein matrix. Terrell et al. (1981) reported decreased moisture loss (from 46.7% to 30.7%) during cooking of beef clod muscle with addition of 2.5% salt. Whiting (1984) found that salt reduction (from 2.5% to 1.5%) resulted in increased water release from frankfurters.

The effects of fat concentration on juiciness could be separated into two distinct phases: above and below 1.3% salt. Reducing salt $< 1.3\%$ resulted in incomplete protein extraction and water was released during packaging and cooking. This condition increased the importance of fat as a contributor to juiciness. Increasing fat content at salt $> 1.3\%$ decreased juiciness scores. As fat content decreased, water was added to the formulation. It appeared that panelists related moisture content to juiciness. Fat concentration may contribute to juiciness only when moisture content is lower.

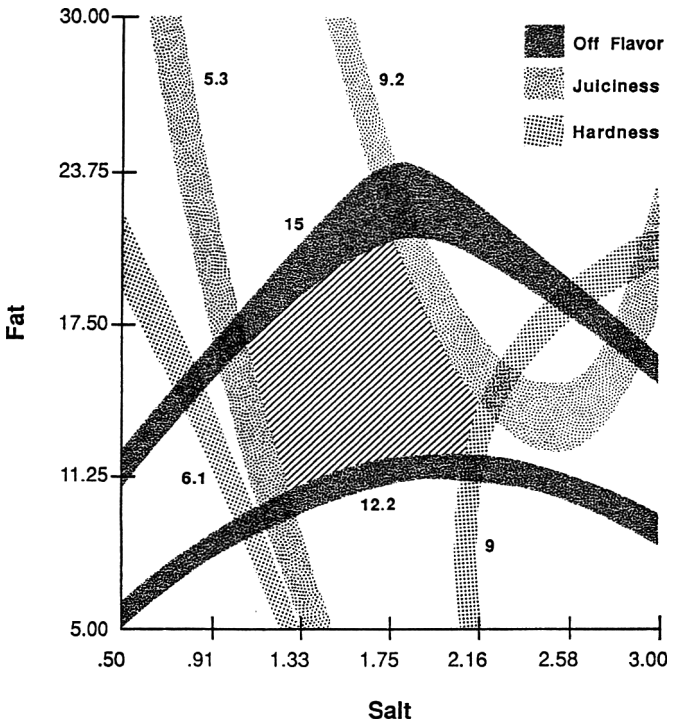


Fig. 10—Effect of fat and salt levels on hardness, juiciness, and off-flavor intensity at pH 6.0. Area shaded falls within 1 standard deviation of the mean for each sensory characteristic.

Most of the variation in frankfurter saltiness was attributed to changes in salt concentration (Fig. 7). Matlock (1983) reported that saltiness of pork sausage patties decreased with levels of salt from 1.5% to 0% salt. Fat and moisture concentrations appear to have limited effects on perceived saltiness.

Flavor intensity of frankfurters was influenced by salt concentration (Fig. 8). As salt increased, flavor intensity increased. These results may be related to flavor enhancement (Gillette, 1985). Fat level had a limited effect on flavor intensity. Fat is a component of meat flavor; however, at fat levels above 5% its influence on flavor intensity appeared to be minimal. Flavor intensity of cured/smoked meat includes salty flavor; the contribution of cooked lean and fat may contribute less to overall flavor intensity because of the high contribution of saltiness. Fat content had more influence than salt concentration on off-flavor intensity (Fig. 9). As fat increased, less off-flavor was perceived at constant salt levels.

At pH 6.0, pH-altering additives (sodium bicarbonate and lactic acid) were not included and no off-flavors were observed. When these additives were included, they produced off-flavors that were not characteristic of commercial frankfurters (data not shown). Bechtel et al. (1989), investigated physical and sensory characteristics of frankfurters produced with various levels of potassium and sodium bicarbonate. They found that adding 0.25% sodium bicarbonate increased off-flavor intensity. The level of sodium bicarbonate to increase pH from 6.0 to 7.0 in our study was 0.4%.

A composite of effects of salt and fat levels on hardness, juiciness, and off-flavor intensity was developed (Fig. 10). For reference, acceptable frankfurters were defined as those products within one standard deviation of the mean for each sensory characteristic of commercial frankfurters. The area within the contours for each sensory characteristic represents the limits of fat and salt combinations at which acceptable frankfurters could be manufactured (Matulis et al., 1994). An acceptable frankfurter (hardness, juiciness, and off-flavor intensity) should be produced with 11.25% fat and 1.33% salt (Fig. 7). These levels represent a 62.5% reduction in fat and a 46.8% reduction in salt content compared to commercial frankfurters (30% fat and 2.5% salt).

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Sensory Characteristics of Frankfurters as Affected by Salt, Fat, Soy Protein, and Carrageenan

RICHARD J. MATULIS, FLOYD K. McKEITH, JOHN W. SUTHERLAND, and M. SUSAN BREWER

ABSTRACT

Continuous variables, salt, fat, and either soy protein or iota-carrageenan, were evaluated using response surface methods to formulate a lower salt/lower fat frankfurter with acceptable palatability attributes. Soy protein increased hardness and off-flavor, and decreased juiciness, saltiness, and flavor intensity. Soy protein should be added to frankfurters at < 3.0% to minimize this effect. Carrageenan increased hardness at low salt concentrations (below 1.7%) and decreased juiciness at fat concentrations > 15%. Flavor intensity increased as carrageenan and fat level increased at 1.3% salt. At 1.65% salt, off-flavor intensity decreased as fat content increased and carrageenan decreased.

Key Words: frankfurters, beef, carrageenan, soy protein, low salt

INTRODUCTION

CONCERN OVER DIETARY FAT AND SODIUM in the diet has caused meat processors to assess the effects of fat and salt reduction in emulsified sausage products (Matulis et al., 1994a). Fat and salt contribute to sensory properties characteristic of frankfurters. Reduced fat and salt frankfurters may have widespread appeal to health-conscious consumers. According to USDA (1988) guidelines, water may replace fat in a formulation on an equal weight basis. Increased amounts of water may affect sensory texture and juiciness of frankfurters, as well as specific flavor intensities. Reducing salt content decreases protein extraction and water binding in addition to changing palatability attributes, such as saltiness, flavor intensity, and juiciness. Some water-binding properties that may be lessened by salt reduction may be partially compensated by adding soy protein or carrageenan.

Soy protein has been added to processed meats as a binder. Soy protein in an emulsified meat product is limited by the USDA (1988) to 3.5% flour or 2.0% soy protein isolate. In finely ground meat products, soy protein gels to form a matrix entrapping moisture and lipid droplets, resulting in improved emulsion stability (Mittal and Usborne, 1985). However, soy protein in meat products may result in "cereal like" off-flavors (Williams and Zabik, 1975) and discoloration if product is ground above 10°C (Mittal and Usborne, 1985).

Peng et al. (1982a,b), evaluated interactions between glycinin, a major soy protein and myosin in a model system. They concluded that soy proteins may act as diluents to interactions among myosin heavy chains. Soy proteins may reduce rubbery texture of low-fat frankfurters by hindering protein-protein interactions.

Sofos et al. (1977) investigated the effects of replacing lean meat with textured soy protein (TSP) and soy protein isolate (SPI) by 25% to 50% of raw batter weight in emulsified sausages. They reported that hydrated TSP added above 25% resulted in emulsion in stability. Inclusion of SPI alleviated stability problems; however, the texture (instrumental) became crumbly. Sofos and Allen (1977) investigated the effect of substituting TSP and SPI for fat to improve emulsified texture in low-fat sausages. As fat level decreased and TSP level increased, Instron hardness increased. The 10% fat product was very hard (instrumental) and could be softened only by substituting some lean beef with pork. They concluded that soy protein could partially replace fat and maintain acceptable sensory texture.

Foegeding and Ramsey (1986) investigated the effects of adding various gums to low-fat (11–12%) frankfurters. In emulsions cooked to 70°C, methylcellulose was the only gum resulting in lower yields. Addition of xanthan gum decreased springiness, hardness, and force to fracture. Frankfurters formulated to contain 26.9% fat (control), 12% fat (low fat), and low-fat frankfurters with added iota-carrageenan and kappa-carrageenan gums were similar for overall acceptability despite differences in textural properties. Results indicated that iota-carrageenan may alleviate toughness frequently occurring in low-fat emulsified sausages.

Matulis et al. (1994a) reported that commercially available frankfurters had a wide range of textural and sensory characteristics. Inclusion of the mean ± one standard deviation for each characteristic included about 66% of the products assessed. Because those products were commercially successful, they presumed that they were acceptable.

Our objective was to evaluate the effects of fat, salt, and soy protein or iota-carrageenan on sensory characteristics of frankfurters.

MATERIALS & METHODS

Experimental design

Continuous variables of salt, fat, and either soy protein or iota-carrageenan, were evaluated simultaneously in attempts to formulate lower salt/lower fat frankfurters with acceptable palatability attributes. A central composite design was used to evaluate results using response surface methods (Box et al., 1978). For each experiment, 24 batches of frankfurters were manufactured in a 2 (salt levels) × 2 (fat levels) × 3 (soy protein or iota-carrageenan levels) factorial design.

Table 1—Experimental design for response surface model for characteristics of frankfurters with varying salt, fat, and soy protein (treatment 1)

Run	X_1	X_2	X_3
1	−1	−1	−1
2	+1	−1	−1
3	−1	+1	−1
4	+1	+1	−1
5	−1	−1	0
6	+1	−1	0
7	−1	+1	0
8	+1	+1	0
9	−1	−1	+1
10	+1	−1	+1
11	−1	+1	+1
12	+1	+1	+1

	Coded matrix variables		
Treatment	−1	0	+1
X_1 (% fat)	12.0	—	18.0
X_2 (% salt)	1.3	—	2.0
X_3 (% soy)	0	1.5	3.0

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Table 2—Formulations for frankfurters with varying salt, fat, and soy protein (treatment 1)

Ingredient, g	Order of formulation											
	1	2	3	4	5	6	7	8	9	10	11	12
Lean	2983	2924	2983	2924	2983	2924	2983	2924	2983	2924	2983	2984
Fat	640	1031	640	1031	640	1031	640	1031	640	1031	640	1031
Water	2220	1889	2182	1851	2138	1807	2100	1769	2057	1725	2020	1688
Salt	71.5	71.5	110.0	110.0	71.5	71.5	110.0	110.0	71.5	71.5	110.0	110.0
Spice mix	27.5	27.5	27.5	27.5	27.5	27.5	27.5	27.5	27.5	27.5	27.5	27.5
Prague powder	13.7	13.7	13.7	13.7	13.7	13.7	13.7	13.7	13.7	13.7	13.7	13.7
Sodium erythorbate	3.0	3.0	3.0	3.0	3.0	3.0	3.0	3.0	3.0	3.0	3.0	3.0
NFDM[a]	198.0	198.0	198.0	198.0	198.0	198.0	198.0	198.0	198.0	198.0	198.0	198.0
Soy protein	—	—	—	—	82.5	82.5	82.5	82.5	165.0	165.0	165.0	165.0

[a] Nonfat dry milk

Table 3—Experimental design for response surface model for characteristics of frankfurters with varying fat, salt, and carrageenan (treatment 2)

Run	X_1	X_2	X_3
1	−1	−1	−1
2	+1	−1	−1
3	−1	+1	−1
4	+1	+1	−1
5	−1	−1	0
6	+1	−1	0
7	−1	+1	0
8	+1	+1	0
9	−1	−1	+1
10	+1	−1	+1
11	−1	+1	+1
12	+1	+1	+1

	Coded matrix variables		
Treatment	−1	0	+1
X_1 (% fat)	12.0	—	18.0
X_2 (% salt)	1.3	—	2.0
X_3 (% gum)	0	0.2	0.4

Treatment 1. Based on results of earlier work (Matulis et al., 1994b), salt concentrations of 1.3% and 2.0%, fat levels of 12% and 18%, and pH 6.0 were selected. These levels represented the lowest ranges of fat and salt that could produce a frankfurter with acceptable sensory characteristics (Matulis et al., 1994a,b). Three levels of soy protein (0%, 1.5%, and 3.0% dry basis) were used, resulting in a 2 × 2 × 3 factorial design (Table 1). Fat and salt levels in this treatment are shown in Table 2.

Treatment 2. Salt concentrations of 1.3% and 2.0%, fat levels of 12% and 18%, and pH 6.0 were used. Two levels of carrageenan gum (0%, 0.2%, and 0.4%) were evaluated, resulting in a 2 × 2 × 3 factorial experimental design (Table 3). Fat and salt levels in this treatment are shown in Table 4.

Frankfurter production

Frankfurters were manufactured as described by Matulis et al. (1994b). The order of manufacture was from a no soy- or carrageenan-added formulation to highest (3.0%) soy- or (0.4%) carrageenan-containing formulation, and from lowest (1.3%) salt content to highest (2.0%) salt content, to minimize cross-contamination among batches.

Chemical analysis and sensory evaluation

Ultimate pH and proximate analyses for fat and moisture contents were determined as described by Matulis et al. (1994b). Sensory evaluation of frankfurters was conducted as described by Matulis et al. (1994b).

Statistical analysis

Statistical analyses conducted are described in Matulis et al. (1994b). Bartlett's test for homogeneity of variance (Bartlett, 1947) was conducted to determine if the variance was the same for all 6 panelists × 12 tests (72 panelist/test combinations [2 trials/combination/panelist]) within soy or gum group. If the hypothesis of a constant variance was rejected, then the replicate measures that differed by >7.5 cm out of 15 cm were discarded. By sensory characteristic, the number of data points discarded for soy-containing samples was hardness-2, cohesiveness-2, juiciness-7, saltiness-1, flavor intensity-2, and off-flavor intensity-5. The number of data points discarded for carrageenan gum-containing samples was cohesiveness-1; no data points were discarded for other characteristics.

RESULTS & DISCUSSION

THE INFLUENCES OF SALT, FAT, SOY PROTEIN, and carrageenan on sensory characteristics of frankfurters were expressed as contour plots. These were two-dimensional plots with two continuous variables on the X and Y axes, and the third variable held constant. By changing the level of the third (constant) value, or by placing that variable on an axis and holding a different variable constant, relationships among the three could be readily visualized.

Treatment 1

The purpose of this treatment was to evaluate effects of altering salt, fat, and soy protein concentration on sensory characteristics. The pH was held constant at 6.0; salt concentration ranged from 1.3% to 2.0%, and fat ranged from 12% to 18%. Upper limits of salt and fat (2.0% salt, 18% fat) represented 20% reduction in salt and 40% in fat, compared to commercial products. Soy protein concentrations (0%, 1.5%, and 3.0%) were based on industrial usage levels and the ability of soy protein to bind water.

For all response surface graphs the variable (salt or fat) not displayed on the X axis was held constant. The variable held constant was the one with least interaction with soy protein for the characteristic evaluated.

Table 4—Formulations for frankfurters with varying fat, salt, and carrageenan (treatment 1)

Ingredient (g)	Order of formulation											
	1	2	3	4	5	6	7	8	9	10	11	12
Lean	2983	2924	2983	2924	2983	2924	2983	2924	2983	2924	2983	2924
Fat	640	1031	640	1031	640	1031	640	1031	640	1031	640	1031
Water	2200	1889	2182	1851	2209	1878	2171	1840	2198	1867	2160	1829
Salt	71.5	71.5	110.0	110.0	71.5	71.5	110.0	110.0	71.5	71.5	110.0	110.0
Spice mix	27.5	27.5	27.5	27.5	27.5	27.5	27.5	27.5	27.5	27.5	27.5	27.5
Prague powder	13.7	13.7	13.7	13.7	13.7	13.7	13.7	13.7	13.7	13.7	13.7	13.7
Sodium erythorbate	3.0	3.0	3.0	3.0	3.0	3.0	3.0	3.0	3.0	3.0	3.0	3.0
NFDM	198.0	198.0	198.0	198.0	198.0	198.0	198.0	198.0	198.0	198.0	198.0	198.0
Carrageenan	—	—	—	—	11.0	11.0	11.0	11.0	22.0	22.0	22.0	22.0

Table 5—Bartlett's test for variance homogeneity for characteristics of frankfurters with varying fat, salt, and soy protein (treatment 1)

Characteristic	Calc chi-squared value[a]	Hypothesis rejected[b]
Hardness	99.44	No
Cohesiveness	67.21	No
Juiciness	114.13	Yes
Saltiness	91.63	No
Flavor intensity	108.55	Yes
Off-flavor intensity	196.24	Yes

[a] Calculated chi-square value from Bartlett's test.
[b] If the calculated chi-square value is greater than chi-square (71, 0.99) = 100, the assumption of a constant variance is refuted.

Table 7—Bartlett's test for variance homogeneity for characteristics of frankfurters with varying fat, salt, and carrageenan (treatment 2)

Characteristic	Calc chi-square value[a]	Hypothesis rejected[b]
Hardness	74.27	No
Cohesiveness	71.11	No
Juiciness	83.64	No
Saltiness	58.81	No
Flavor intensity	85.08	No
Off-flavor intensity	150.68	Yes

[a] Calculated chi-square value from Bartlett's test.
[b] If the calculated chi-square value is greater than chi-square (71, 0.99) = 100, the assumption of a constant variance is refuted.

Bartlett's (1947) test (Table 5) showed panelists were inconsistent when evaluating juiciness, flavor intensity, and off-flavor intensity. Inconsistent data were removed so that variance homogeneity could be assumed for remaining tests. Regression equations for Treatment 1 are shown in Table 6.

The combined effects of soy protein and fat content on sensory hardness with salt concentration constant at 1.65% (the intermediate level for this treatment) were compared (Fig. 1). Hardness increased as soy protein increased for all fat concentrations. Soy protein can bind up to seven times its weight in water. As the percentage of soy protein increased, percentage of water decreased (a direct substitution); reduction in water content may have contributed to hardness increases. Sofos et al. (1977) and Decker et al. (1986) reported that soy protein added to low-fat frankfurters decreased Instron hardness when hydrated soy protein was substituted for meat in the formulation. However, neither study investigated addition of soy protein while maintaining a constant muscle protein level.

Hardness decreased with increasing fat. This did not confirm results of our earlier work (Matulis et al., 1994b); however, the range of fat levels in the present study was lower than in our earlier study. Therefore the magnitude of the response may have been diminished. Soy protein may bind water allowing fat to soften texture by reducing protein-protein interactions (Rakosky, 1970).

Cohesiveness decreased as soy protein increased when salt concentration was held constant (Fig. 2). Soy protein addition to high water frankfurters was expected to increase cohesiveness because of its water-binding. Soy protein addition may have resulted in a more crumbly texture detected by panelists as cohesiveness reduction. Cohesiveness as judged by panelists was not assessed consistently. This may have been due to lack of training, or because cohesiveness was confounded by changes in springiness and firmness which were not assessed. Cohesiveness increased with increasing fat.

As soy protein content increased, juiciness scores decreased (Fig. 3). Decreased juiciness may be due to soy protein binding free water resulting in reduced moisture release on initial bite. In addition, as soy protein content increased, it replaced water on an equal weight basis. As fat content increased, juiciness scores decreased which confirmed our earlier findings (Matulis et al., 1994b). The decrease in juiciness may have been due to replacement of water with fat on an equal weight basis.

As soy protein increased, frankfurter saltiness decreased (Fig. 4). The flavors produced by soy protein may have masked the intensity of other flavor components. The juiciness reduction associated with increased levels of soy protein may have been responsible for saltiness reduction. A similar relationship between soy protein and flavor intensity was seen (Fig. 5). Decker et al. (1986) reported a decrease in "meaty" flavor as isolated soy protein increased in frankfurters; however, soy protein replaced muscle protein in their formulations. They also reported that soy protein had no effect on frankfurter "spiciness."

Off-flavor intensity increased as soy protein concentration increased (Fig. 6). When 3.0% soy protein was added, the mean off-flavor score was 12 (1 = intense, 15 = bland) across all fat levels. We reported that the minimum acceptable off-flavor score for frankfurters was 12.2 cm (when 15 = none) (Matulis et al., 1994a). Soy protein should be limited to < 3.0% to avoid undesirable off-flavors. When using high levels of texturized soy protein (25% hydrated 1:2) to substitute for lean beef in frankfurters, Sofos et al. (1977) reported that the soy product produced an unacceptable flavor. They recommended altering the seasoning of frankfurters containing soy protein to alleviate off-flavors. Seasoning was not altered in our study because changes could affect saltiness, water binding, and flavor intensity which would affect other variables.

As fat level increased, off-flavor intensity increased. These results did not confirm our earlier results (Matulis et al., 1994b), which indicated that off-flavor intensity increased with decreasing fat levels. The range of fat levels was more narrow in this than in the previous study. When no soy protein was added, all scores were acceptable (lowest score 13.5 at 20% fat.)

Treatment 2

This treatment was designed to evaluate concurrent effects of fat, salt, and iota-carrageenan on sensory characteristics. Salt ranged from 1.3% to 2.0% and fat from 12% to 20%. Gum levels (0% to 0.5%) were based on studies by Foegeding and Ramsey (1986) and industrial usage levels. Bartlett's test (Table 7) showed panelists were inconsistent when evaluating off-flavor intensity. The regression equations developed for Treatment 2 are shown in Table 8.

Carrageenan had little effect on sensory hardness when salt was constant (Fig. 7). However, increasing carrageenan resulted

Table 6—Regression coefficients to predict sensory characteristics of low fat/low salt frankfurters formulated with soy protein[a]

Sensory characteristic	b_0[b]	b_1	b_2	b_3	b_{12}	b_{13}	b_{23}	b_{33}
Hardness	4.55*	−0.47	+0.14	+1.03*	+0.06	+0.36	+0.01	+2.79*
Cohesiveness	8.73*	+0.09	+0.03	−0.18	+0.33	+0.11	−0.31	−1.28*
Juiciness	6.09*	−0.49	+0.46	−0.29	−0.61*	−0.60	−1.03*	+1.34*
Saltiness	4.96*	+0.20	+1.07*	−0.60*	−0.09	−0.14	−0.23	+0.79
Flavor intensity	5.49*	−0.06	+1.03*	−0.58	+0.06	−0.07	−0.10	+0.74
Off-flavor intensity	13.45*	+0.04	−0.09	−0.76*	−0.09	+0.25	−0.18	+0.38

[a] Regression coefficients to fit the equation $y = B_0 + B_1X_1 + B_2X_2 + B_3X_3 + B_{12}X_1X_2 + B_{13}X_1X_3 + B_{23}X_2X_3 + B_{33}X_3^2$ + error, where X_1 = % fat, X_2 = % salt, and X_3 = % soy protein.
[b] b_0 is the least squares estimate of B_0.
* $P < 0.05$.

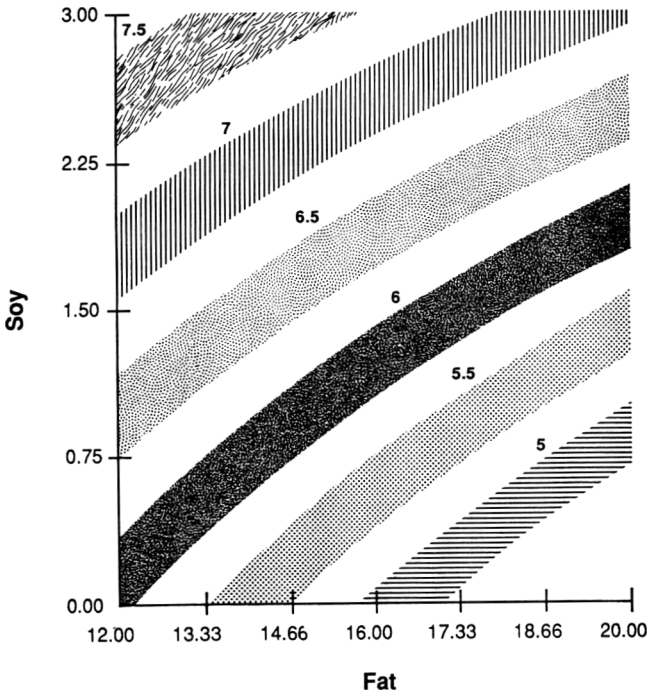


Fig. 1—Effect of soy protein and fat (%) on sensory hardness at 1.65% salt. Response values: 0 = extremely soft, 15 = extremely hard.

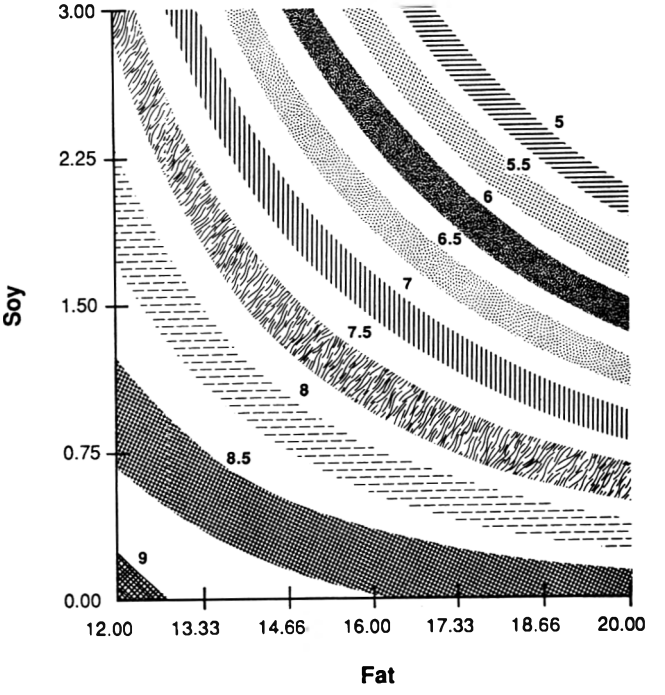


Fig. 3—Effect of soy protein and fat (%) on juiciness at 2% salt. Response values 0 = extremely dry, 15 = extremely moist.

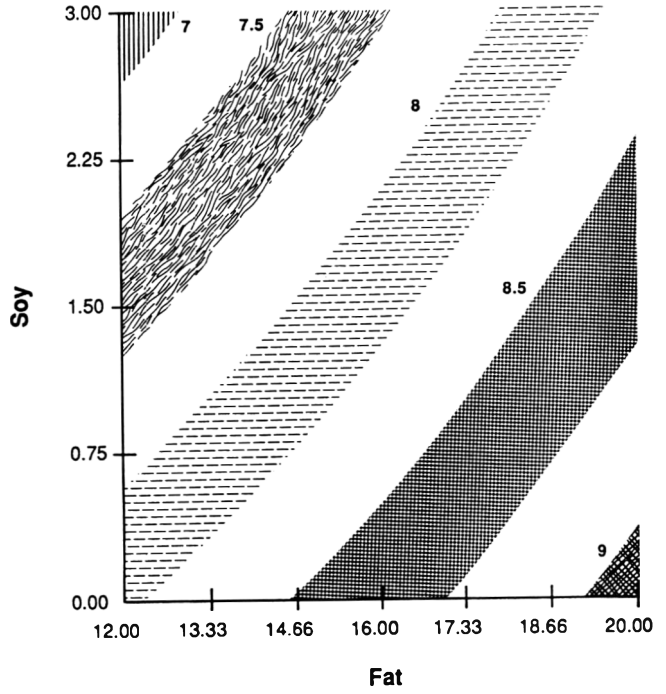


Fig. 2—Effect of soy protein and fat (%) on sensory cohesiveness at 2% salt. Response values: 0 = extremely soft, 15 = extremely cohesive.

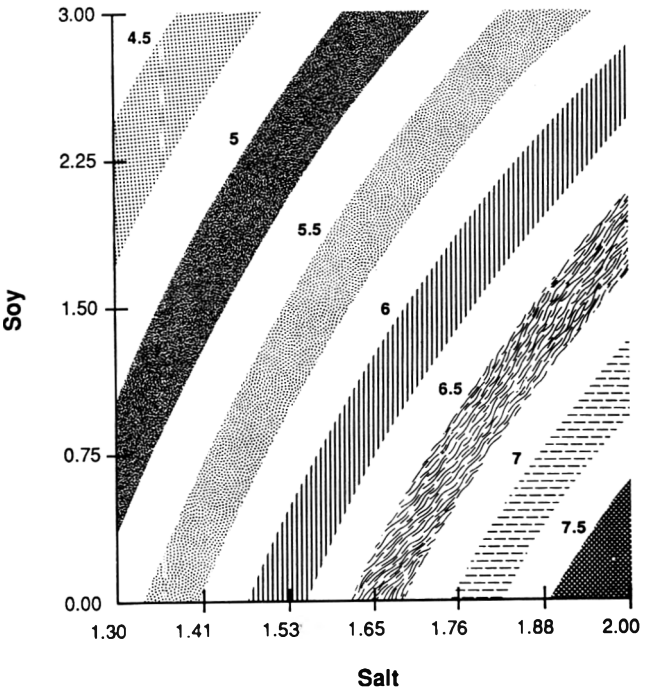


Fig. 4—Effect of soy protein and salt (%) on saltiness at 18% fat. Response values: 0 = extremely bland, 15 = extremely salty.

Table 8—Regression coefficients to predict sensory characteristics of low fat/low salt frankfurters formulated with carrageenan[a]

Sensory characteristic	b_0[b]	b_1	b_2	b_3	b_{12}	b_{13}	b_{23}	b_{33}
Hardness	7.86*	+0.20	+0.02	+0.25	+0.54*	+0.19	−0.28	−0.54*
Cohesiveness	7.39*	+0.63*	+0.23	+0.27	+0.13	−0.09	+0.09	−0.48
Juiciness	8.61*	−0.47*	+0.18	−0.28	−0.24	−0.71*	−0.21	−0.69*
Saltiness	8.01*	−0.41*	+0.59*	+0.16	−0.00	−0.09	−0.25	−0.25
Flavor intensity	9.20*	−0.41*	+0.52*	+0.31	+0.13	−0.05	−0.07	−0.54*
Off-flavor intensity	14.07*	+0.20	+0.28	−0.68*	−0.08	−0.07	+0.55*	−0.86*

[a] Regression coefficients to fit the equation $y = B_0 + B_1X_1 + B_2X_2 + B_3X_3 + B_{12}X_1X_2 + B_{13}X_1X_3 + B_{23}X_2X_3 + B_{33}X_3^2$ + error, where X_1 = % fat, X_2 = % salt, and X_3 = % carrageenan.

[b] b_0 is the least squares estimate of B_0.

* $P < 0.05$.

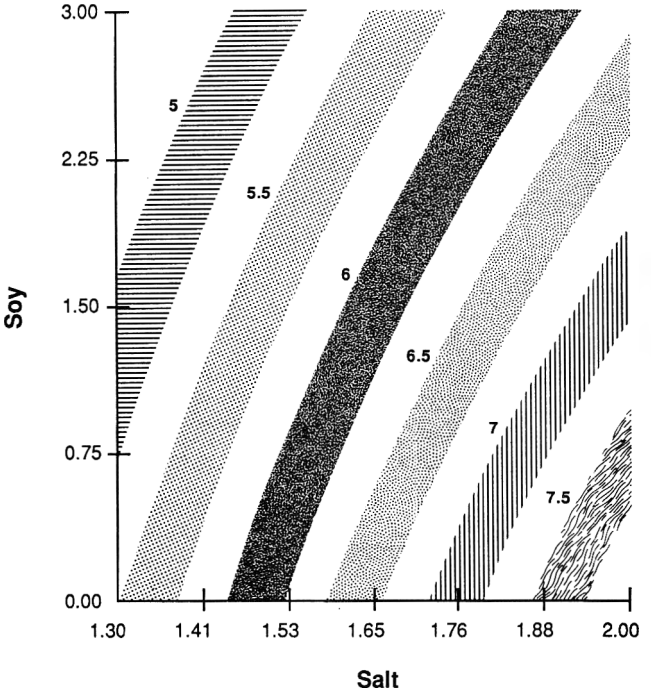


Fig. 5—Effect of soy protein and salt (%) on flavor intensity at 18% fat. Response values: 0 = extremely bland, 15 = extremely intense.

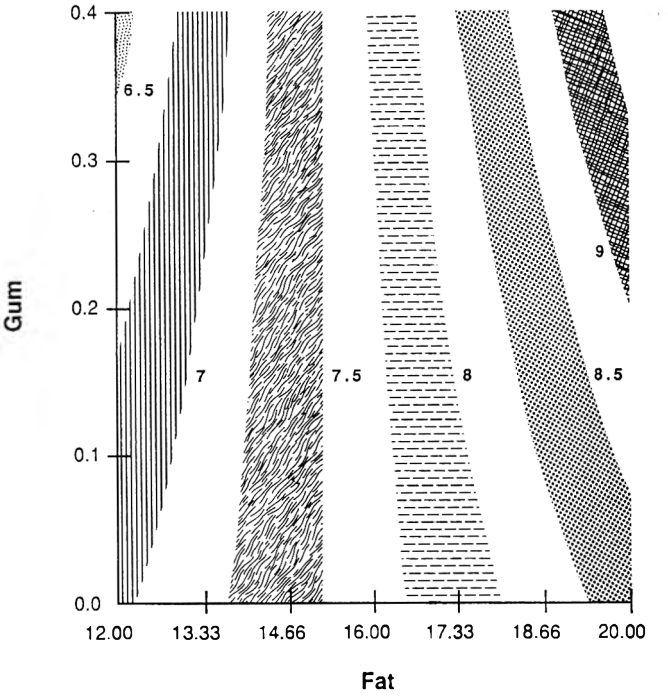


Fig. 7—Effect of carrageenan gum and fat (%) on sensory hardness at 2% salt. Response values: 0 = extremely soft, 15 = extremely hard.

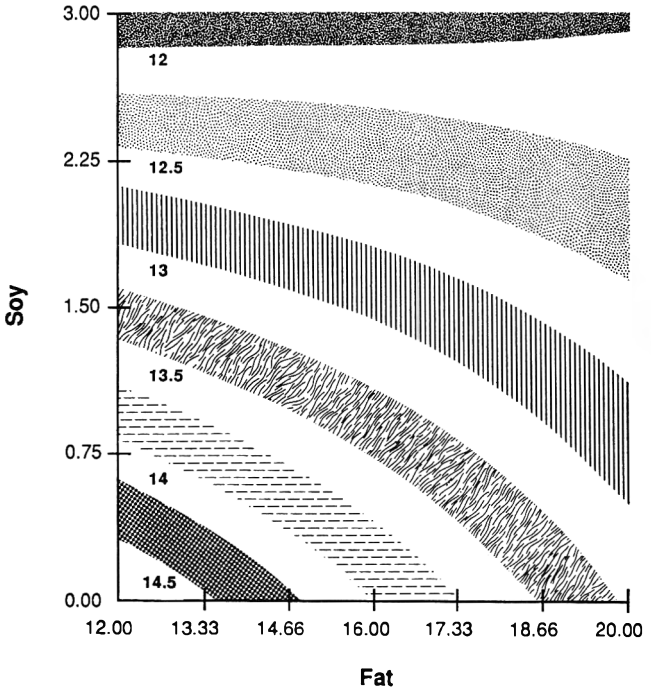


Fig. 6—Effect of soy protein and fat (%) on off-flavor intensity at 1.65% salt. Response values: 0 = extremely intense, 15 = extremely bland.

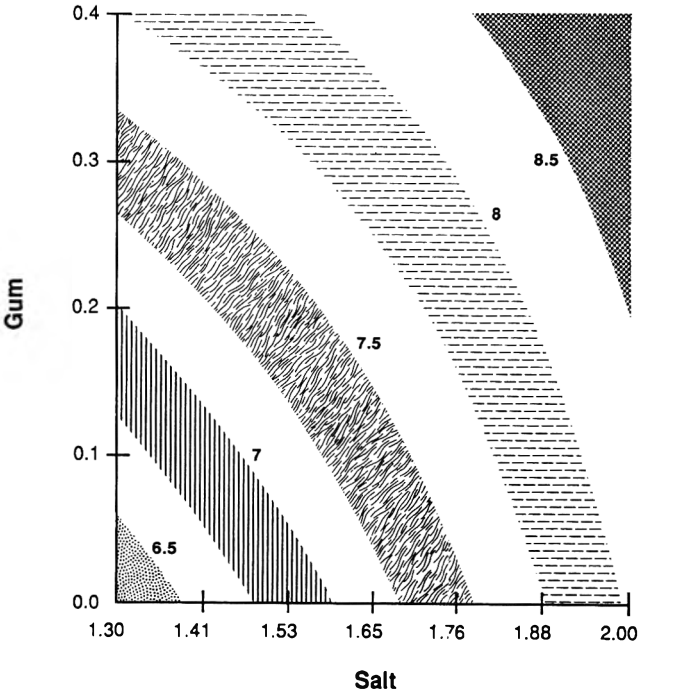


Fig. 8—Effect of carrageenan gum and salt (%) on sensory hardness at 18% fat. Response values: 0 = extremely soft, 15 = extremely hard.

in increased hardness at lower salt (Fig. 8). Foegeding and Ramsey (1986) found that textural characteristics of low-fat (10%), high moisture frankfurters made with iota-carrageenan gums were not different from low-fat, high moisture controls. However, salt concentration was higher (2.35%) than salt levels in our present study (between 1.3% and 2.0%). Carrageenan may bind excess free water at lower salt, resulting in firmer products. Addition of iota-carrageenan to frankfurters might be most beneficial when salt levels were reduced. Cohesiveness increased as carrageenan level increased (Fig. 9). Fat concentration had more effect on cohesiveness than did carrageenan.

Carrageenan and fat content effects on frankfurter juiciness were compared (Fig. 10). Below 15% fat, all frankfurters rated high juiciness scores (above 8.5 on a 15-point scale); juiciness decreased as carrageenna increased as fat content increased > 15%. Carrageenan binds large quantities of free water; less water is released on initial bite which may influence sensory perceptions of juiciness. However, all frankfurters had juiciness scores within acceptable limits (5.3 to 9.2 on a 15-point scale, Matulis et al., 1994b). Juiciness decreased as fat level increased. These results were consistent with those found in our earlier

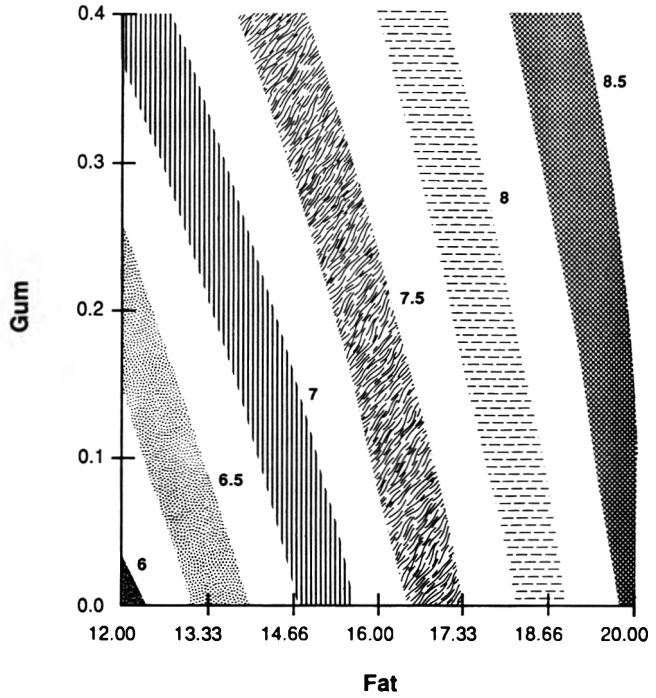


Fig. 9—Effect of carrageenan gum and fat (%) on cohesiveness at 2% salt. Response values: 0 = extremely soft, 15 = extremely cohesive.

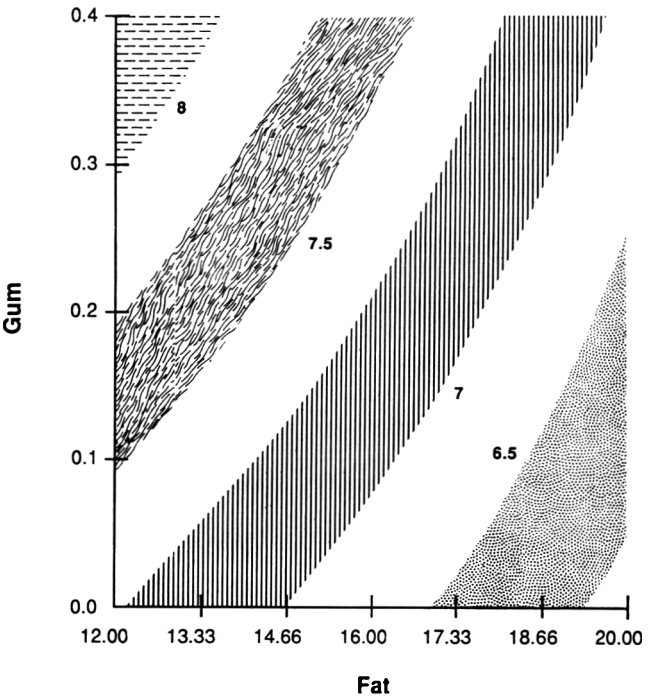


Fig. 11—Effect of carrageenan gum and fat (%) on saltiness at 1.3% salt. Response values: 0 = extremely bland, 15 = extremely salty.

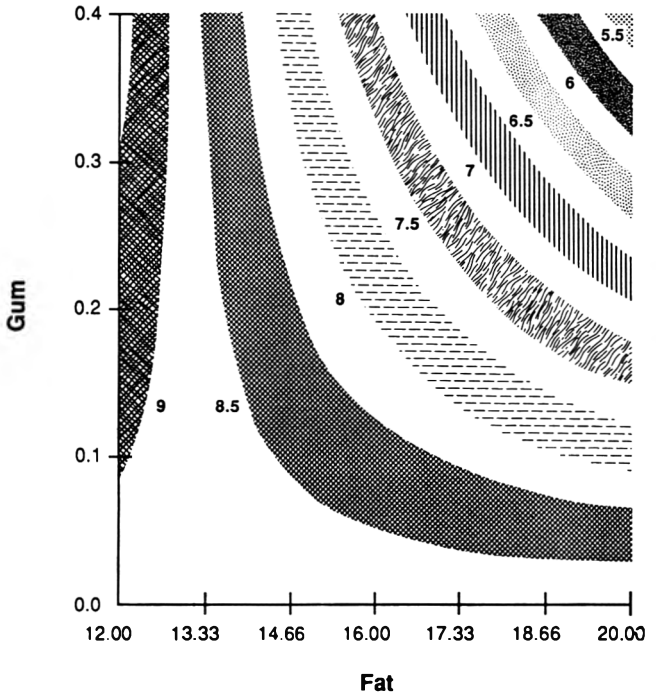


Fig. 10—Effect of carrageenan gum and fat (%) on juiciness at 2% salt. Response values: 0 = extremely dry, 15 = extremely juicy.

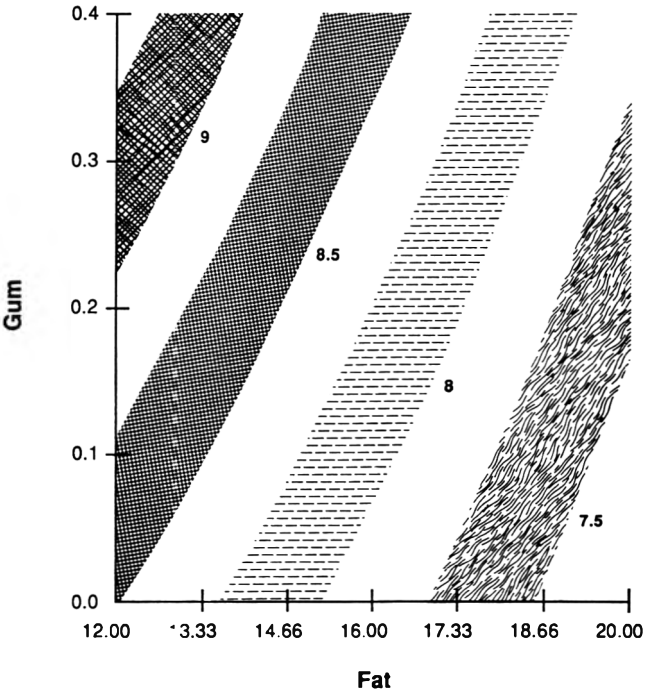


Fig. 12—Effect of carrageenan gum and fat (%) on flavor intensity at 1.3% salt. Response values: 0 = extremely bland, 15 = extremely intense.

studies (Matulis et al., 1994b, and Treatment 1) and may be due to substitution of fat for water on an equal weight basis.

As carrageenan levels increased, saltiness increased and flavor intensity decreased (Fig. 11 and 12). As carrageenan level increased, off-flavor intensity decreased (15 = bland) at all fat levels (Fig. 13). All off-flavor scores were within acceptable levels established previously (Matulis et al., 1994b). Off-flavor intensity increased as fat increased. Foegeding and Ramsey (1986) did not detect differences in preference between frankfurters formulated with iota-carrageenan and controls.

SUMMARY & CONCLUSIONS

ADDITION OF SOY PROTEIN increased sensory hardness of frankfurters at all fat levels; hardness decreased as fat level increased. Soy protein decreased cohesiveness and juiciness while fat increased cohesiveness and juiciness. Soy protein decreased saltiness and increased off-flavor intensity. Soy protein should be limited to < 3%. Carrageenan had little effect on sensory hardness when salt was held constant but increased hardness at lower salt concentrations. Addition of carrageenan to frankfurters may be most beneficial when salt levels are reduced. Fat concentra-

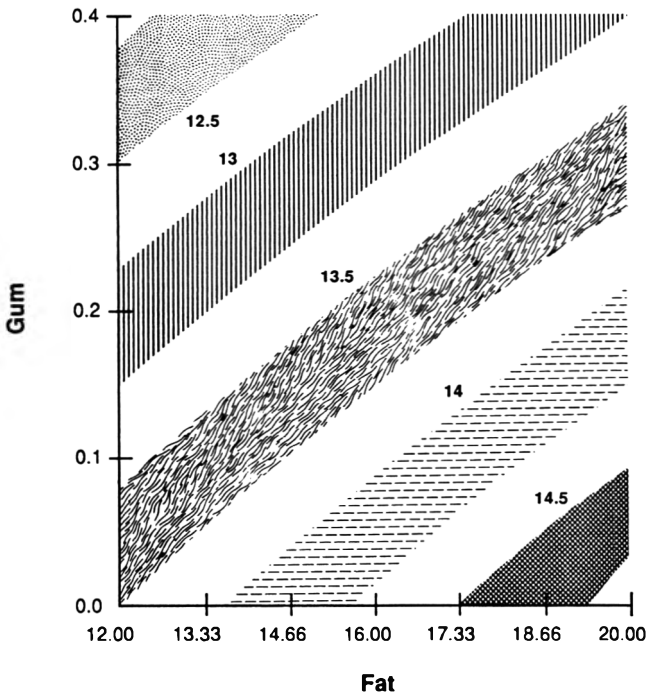


Fig. 13—Effect of carrageenan gum and fat (%) on off-flavor intensity at 1.65% salt. Response values: 0 = extremely intense, 15 = extremely bland.

tion had a greater effect on cohesiveness than did carrageenan. Below 15% fat, all frankfurters received high juiciness scores; juiciness decreased as carrageenan and fat increased. As carrageenan increased, saltiness increased while flavor intensity and off-flavor decreased. Carrageenan appeared to be most effective at low salt and fat concentrations.

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Springiness, cohesiveness, and chewiness were not affected by particle size (Table 3). These results are similar to those of Barbut (1988), who reported that chopping time did not affect springiness and cohesiveness.

Post-mince mixing time did not affect ($P>0.05$) total energy, hardness, peak force, and cohesiveness (Table 2). Frankfurters produced by mixing 15 min tended ($P<0.057$) to be more springy and chewy than those mixed 5 min (Table 2). Sylvia (1992) reported that prolonged mixing time (30 min) did not affect hardness but when post-minced batter was mixed with 30% of the formulation water at 16°C, frankfurters were less firm than controls. Under similar treatments as we used, Sylvia (1992) also reported no difference in springiness, cohesiveness, and chewiness.

CONCLUSIONS

PROCESSORS can manipulate textural properties more through alterations in particle size than through changes in mixing time. However, changes in particle size also may alter product yellowness. Utilizing larger plate sizes may increase the perception of particles which may not be desirable in some finely comminuted sausages.

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Formulation and Chopping Temperature Effects on Beef Frankfurters

J.L. HENSLEY and L.W. HAND

ABSTRACT

Frankfurters were manufactured using four fat and added water (AW) formulations (10% fat/30% AW; 15%/25%; 20%/20%; 30%/10%) and processed at chopping temperatures 9, 12, or 15°C. The batters were stuffed into cellulose casings, thermally processed, chilled and vacuum-packaged. Frankfurters were analyzed for proximate composition, textural properties and purge. No differences occurred among treatments for processing yield (89.8% ± 1.83). Frankfurters chopped to 12°C had the highest ($P < 0.05$) Kramer peak force values. As expected, purge increased in all treatments as storage time increased ($P < 0.05$). As AW increased, hardness and cohesiveness decreased and purge increased. Water level and fat reduction were the most critical factors affecting quality.

Key Words: beef, frankfurters, texture, purge loss, cohesiveness

INTRODUCTION

INCREASED CONSUMER DEMAND for low-fat products creates increased research demands for the red meat industry. Fat content in processed meat products can be readily reduced through formulation with leaner meats, but this increases cost. It may also lessen product quality (Hand et al., 1987). Rust and Olson (1988) reported that when fat of a meat product was reduced below 15%, the quality of the product changed considerably, especially the texture. Fat reduction with 10% or less added water (AW) has resulted in products that were firmer, more rubbery and less juicy (Rongey and Bratzler, 1966; Decker et al., 1986; Hand et al., 1987). USDA labeling changes (Federal Register, 1988) allow the red meat industry opportunities to produce cooked sausage products, primarily frankfurters and bologna, that are low in fat yet still economical to manufacture. The combined total fat and added water content cannot exceed 40%, whereas the traditional level was 30% fat and 10% AW. Increasing the water level beyond traditional levels tends to diminish the stability of the meat system. This partially explains the excess purge, high cooking loss and soft texture (Claus et al., 1989, 1990) usually reported for low-fat beef products in which water has replaced fat. Water binding has become as important as fat binding in frankfurter systems (Rust and Olson, 1988), even though Morrison et al. (1971) indicated that moisture binding was the most critical factor for product stability. Water binding and product stability are affected by many variables, one of the more critical is chopping temperature.

Several researchers have investigated the effects of changing temperature on product stability and generally concluded that optimal final chopping temperature was 12–22°C. Brown and Toledo (1975) concluded that maximum binding occurred in the range 15–22°C. Helmer and Saffle (1963) found stable emulsions chopped to 15.7°C. Klettner (1987) reviewed comminution techniques for frankfurter-type sausages and concluded that the final cutter temperature should be no higher than 13°C. Several researchers (Heidtmann, 1962; Janke, 1977; Reichert, 1982) concluded that the final product temperature should be below 15°C for frankfurter-type sausages. Klettner (1987) reported that Ambrosiadis and Wirth (1983) had indicated the final temperature should be 10–12°C without phosphate and 16–18°C with phosphate. However, since they used combined species (beef and pork) formulations and traditional fat and AW levels, such studies need to be re-examined in low-fat, high-moisture frankfurters.

Endpoint chopping temperatures have been established as critical control points in frankfurter manufacture for maximum protein extraction (Jones and Mandigo, 1982). However, such temperatures were also not related to low-fat, high-moisture systems. Optimum temperatures for maximum solubility of finely ground beef mixtures have been reported between 10°C and 12°C (Gillette et al., 1977; Gadea de Lopez and Hand, 1993). As processors consider reducing fat content by removal of fat and replacement with water, chopping temperatures may be critical to the (<30% fat), higher-moisture (>10% AW) products. Therefore, our objective was to evaluate the effects of different formulations and chopping temperatures on properties of beef frankfurters.

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MATERIALS & METHODS

Frankfurter manufacture

Three replications of beef frankfurters were manufactured at four different formulations and three chopping temperatures. Beef frankfurters were chosen as the experimental product because, traditionally, they have not been as reliably consistent in product yields and stability (Schwartz, 1988). U.S. Choice, Yield Grade 3, beef shoulder clods and 50% fat commercial beef trimmings, obtained locally, were individually ground (Biro Mfg., Marblehead, OH) through a 1.27-cm plate. Samples from each were then reground through a 0.32-cm plate and analyzed for proximate composition. Percentages of fat (ether extract), moisture (oven drying) and protein (Kjeldahl) were determined using AOAC (1990) procedures. Appropriate lean, fat and water combinations for 4 fat and added moisture levels were subsequently produced using a least cost formulation program (Least Cost Formulator, Ltd., Virginia Beach, VA). Treatments were formulated for fat and added moisture as follows (% fat/% AW): 10/30; 15/25; 20/20; and 30/10 (Control). They were formulated with expected product yields of 88%. These yield figures were obtained in two preliminary studies to determine the average yields of these formulations. Batch size for each treatment was 11.33 kg.

The percentage of added moisture for initial mixing (26%) was held constant among all treatments. This percentage was calculated by dividing the amount of added moisture by the amount of lean formulated for the 30/10 treatment. The appropriate amount of water was mixed with the lean portion (4.4°C ± 2°), 2% salt, 1.44% spices (A.C. Legg, Birmingham, AL), 0.5% sodium tripolyphosphate, 0.055% sodium erythorbate (550 p.p.m.), and 0.0156% sodium nitrite (156 ppm) in a six-blade vacuum bowl chopper (K64 Seydelmann, Robert Reiser, Canton, MA) for 30 sec on low speed without vacuum. The fat portion (4.4 ± 2°C) and remaining water were then added. This mixture was chopped 4 min on high speed, with the last 30 sec under vacuum. Chopping times used were developed from two preliminary studies in which the batter was chopped to optimum temperatures indicated by Jones and Mandigo (1982). Chopping time remained constant among all treatments.

All formulations were chopped to and maintained at three chopping temperatures (9°, 12°, and 15°C). These were selected to include the optimal protein solubility temperature (12°C) obtained in a laboratory system by Gadea de Lopez and Hand (1993). Upon reaching the appropriate temperature, crushed solid carbon dioxide (CO_2) was added to

maintain that temperature within ± 1°C for the duration of chopping. All formulation and temperature combinations were replicated three times.

The batter was removed from the chopper, vacuum-stuffed (Robot 500, Vemag, Robert Reiser, Canton, MA) into 32-mm cellulose casings (EZ Peel Nojax, Viskase, Chicago, IL) and heat-processed (1-truck smokehouse, Alkar, Lodi, WI) (Table 1). Frankfurters were allowed to cool 18 hr at 4.4°C ± 2° before cooking yields were obtained by weighing the cooked franks and dividing by the weight of the stuffed raw batter.

Frankfurters were peeled by hand and vacuum-packaged (M855, Multivac, Kansas City, MO), five links per package. Samples from each treatment were frozen at −23°C until proximate analysis and texture data could be obtained. Remaining packages were stored at 4.4 ± 2°C for purge and texture analysis.

Raw batter analysis

Thermal emulsion stability of the raw batter was determined using the method described by Townsend et al. (1971). Raw batter (34g) was placed into a plastic conical cylinder, covered with plastic film (Parafilm, American National Can, Greenwich, CT) and heated in a 48.8°C water bath (Model BKS-350, Gallencamp and Co., Sussex, England) with the temperature raised intermittently after 30 min until internal temperature of the batter reached 68.8°C. One tube in each batch was fitted with a thermometer to record temperature during cooking. Liquid released was decanted into 15-mL graduated centrifuge tubes and centrifuged (Model J-6M, Beckman Instruments, Palo Alto, CA) for 1 min at 2.68 × *g*. After chilling (4.4°C, 12 hr) volumes of total liquid, fat, gelatinous water and proteinaceous solids were recorded. Total liquid was expressed as percentage of sample weight and recorded as total percentage loss.

Purge analysis

Purge was measured each week for 8 wk. Week 1 was the first week after production. Purge loss was measured on two random packages for each sampling time. Each package was opened and purge was poured through a funnel into a 15-mL graduated centrifuge tube. The frankfurters were then placed in the funnel to drain (1 min) any purge that was on the surface of the franks. Weights of franks were then recorded. The vacuum packages were dried in the smokehouse (38°C, high fan speed) to ensure that any residual purge in the comers of packages was removed; then package weights were recorded. Purge loss was reported as a percentage of content weight.

Textural analyses

Compression values (quadruplicate) were determined by axially compressing 62.5% of the height of the short axis of a 3-cm-long sample (ambient temperature) cut from the center of the frankfurter. This was done for two cycles with an apparatus attached to an Instron Universal Testing Machine (Model 4500, Instron Corp., Canton, MA). The machine was set at a crosshead speed of 500 mm/min and a 1 kN load cell. Values obtained were hardness and cohesiveness (Singh et al., 1985).

Kramer shear values (quadruplicate) were obtained by shearing the short axis of a 4-cm-long sample (ambient temperature) cut from the center of the frankfurter using the Kramer shear attachment for the Instron with a crosshead speed of 100 mm/min and a load cell of 10 kN. Peak load or force and area under the curve (energy) was used to determine shear measurements (Singh et al., 1985).

Statistical analysis

The experimental design was a 4 × 3 factorial arrangement using four formulation treatments and three chopping temperature treatments with three replications. Treatment means and standard errors were calculated using the Statistical Analysis System (SAS Institute, 1985). Data were subjected to analysis of variance to determine significant interactions and main effects. Error terms were pooled for nonsignificant interactions.

Significant interactions ($P < 0.05$) between formulation and temperature, and temperature and days of storage, on purge as well as emulsion stability by formulation and temperature were analyzed using analysis of variance and regression techniques (Steel and Torrie, 1980). Differences in final product proximate analysis as well as significant differences in purge by interaction of formulation and temperature were analyzed using least square means procedures (Steel and Torrie, 1980).

Table 1—Thermal processing schedule for beef frankfurters

Step	Time (min)	Dry bulb (°C)	Wet bulb (°C)	Humidity (%)
Cook	20	57.7	0	—
Smoke cook	35	68.9	46.5	30
Smoke cook	15	80.1	57.7	35
Evacuation	03	—	—	—
Steam cook	05	85.7	—	100
Cold Shower	01	—	—	—

RESULTS & DISCUSSION

Frankfurter composition

Frankfurter finished product compositional means and standard errors were compared (Table 2). Formulation had the greatest effect on frankfurter composition. As expected, moisture levels were different ($P < 0.05$) between all treatments. The 10% fat/30% AW treatment had the highest amount of moisture, while the 30/10 had the lowest. Although all treatments were formulated to the same protein content, the 10/30 frankfurters had a slightly higher ($P < 0.05$) protein content than the other treatments. They were also below the AW target and this may explain the protein content difference. This is a further indication of the difficulty in maintaining AW levels in low-fat frankfurters such as Claus et al. (1989) reported in bologna products. In examining the AW and 40% rule values, the frankfurters achieved the desired targets with the possible exception of the 10/30 samples. Thus the frankfurters achieved formulation targets within reasonable deviations.

Percentage fat was affected by interaction of formulation and chopping temperature (see regression curves Fig. 1). The 10/30 formulation had higher fat contents as processing temperatures increased. The 15/25 and the 20/20 samples were not influenced ($P > 0.05$) by chopping temperature. The 30/10 formulation had the highest fat content when chopped to 12°C. This may indicate that chopping at 12°C bound more fat in the higher-fat system. Gadea de Lopez and Hand (1993) reported optimal protein extraction in this temperature range. However, we expected a difference in yield to coincide with this difference. Yield was not different ($P > 0.05$) among formulation or chopping temperature (mean = 89.8%, standard error = 1.83). All treatments achieved higher yields than in preliminary studies which were chopped using the same time increments, but without temperature controls. Control of chopping temperature appears to have been beneficial with all treatments.

Raw batter stability

Thermal emulsion raw batter stability was influenced by formulation (Table 3). The 15/25 batter had the greatest ($P < 0.05$) total loss expressed as percentage, while the 20/20 raw batter was the most stable ($P < 0.05$). This was in contrast to frankfurter yield data which showed no difference ($P < 0.05$) among treatments. This may reflect differences when comparing model systems (raw batter stability) to pilot plant processing systems.

Textural analyses

Kramer shear energy (area under the curve) was not different ($P > 0.05$) for any treatments (formulation or chopping temperature). Kramer shear peak force was influenced by both the main effects of formulation and chopping temperature (Tables 3 and 4). Although the magnitude of difference was slight, the 15/25 frankfurters were firmest with the greatest ($P < 0.05$) peak force among all treatments. Frankfurters chopped to 12°C had the highest ($P < 0.05$) peak force values (Table 4). Gadea de Lopez and Hand (1993) found in a model system that the greatest protein extraction from different beef formulations occurred at 12°C. This indicated that 12°C should be the optimum temperature for chopping to achieve a firmer product. Greater protein extraction at a higher temperature would also explain

Table 2—Beef frankfurter composition means and standard errors

Formulation (% fat/% AW)	Moisture	Fat	Protein	AW[a]	40% Rule
10/30	76.0 (0.35)[c]	8.8 (0.47)	12.3 (0.10)[c]	26.7	35.5
15/25	71.6 (0.59)[d]	13.3 (0.60)	11.4 (0.12)[d]	26.2	39.2
20/20	66.4 (0.85)[e]	18.7 (1.07)	11.6 (0.10)[d]	20.1	38.7
30/10	59.1 (0.45)[f]	25.7 (0.77)	11.4 (0.13)[d]	13.3	39.0

[a] Added Water (AW) = Moisture (%) − 4(Protein %).

[b] 40% Rule = AW + Fat % ≤ 40%.

[cdef] Means within a column followed by a different superscript are different ($P < 0.05$). There was a significant formulation × chopping temperature interaction and thus fat is not analyzed by formulation. AW and 40% Rule values are calculated values and were not statistically analyzed. Standard errors are in parentheses.

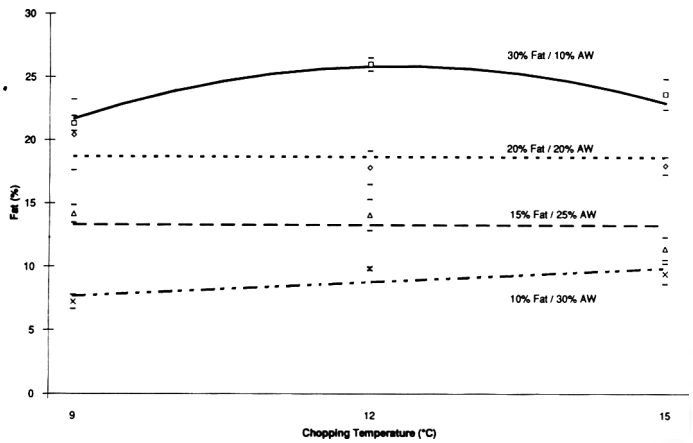


Fig. 1—Frankfurter fat content as influenced by formulation and chopping temperature. Lines are the treatment regression lines. Symbols are treatment means with standard errors.

such results. Jones and Mandigo (1982) reported as temperatures rose from 10° to 16°C protein extraction increased.

Cyclic compression hardness and cohesiveness values were influenced by formulation (Table 3). Hardness is the force necessary to cause deformation (Szczesniak, 1963). This can be associated with the amount of force required to compress a sample between molar teeth (Brady and Hunecke, 1985). Frankfurter hardness increased ($P < 0.05$) from the low-fat formulation (10/30) to the control formulation (30/10). Conversely, as AW increased the hardness decreased. The 30/10 samples were the most cohesive ($P < 0.05$). The greater hardness and cohesiveness in the control product indicated the influence that AW or total moisture had on frankfurter texture. Brady and Hunecke (1985) correlated increased product moisture with decreased Instron compression measurements. Increased fat decreased textural properties when added water was constant (Hand et al., 1987; Morrison et al., 1971). Morrison further indicated that AW had the greatest influence on product stability.

Purge

Formulation and storage interacted to affect purge (Fig. 2). As expected, purge increased for all treatments as storage time increased ($P < 0.05$). The 10/30 treatment resulted in the greatest purge ($P < 0.05$) over time. This was in agreement with Claus et al. (1989) who reported that treatments containing more than 25% added moisture had the greatest purge loss. As expected, the treatment with highest fat level and lowest added moisture level showed the lowest percentage purge, also in confirmation of Claus et al. (1989). Although the other treatments increased in a linear ($P < 0.05$) fashion, the 20/20 increased curvilinearly ($P < 0.05$). With that exception: the greater the AW, the greater ($P < 0.05$) was the rate of purge loss.

Formulation and chopping temperature also influenced percentage purge (Fig. 3). The 10/30 treatment showed a curvilinear response and indicated that the 15°C had the least purge. However, the 15/25 and 30/10 treatments indicated that the 15°C had the greatest increase in purge. The 20/20 samples had a curvilinear response and the 12°C chopping temperature had the least purge. Many companies process to endpoint chopping temperatures typically ≈15°C. They presume that it is necessary to chop at such high temperatures to fully comminute the product, specifically the fat portion. However, often the product may be fully comminuted while maintaining a low temperature, especially with the speed of modern choppers and the use of carbon

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Table 3—Influence of formulation on raw batter stability, Kramer shear force and cyclic compression

Formulation	Raw batter stability	Kramer shear	Cyclic compression	
			Hardness	
(% fat/% AW)	Total loss (%)	Peak force (N)	(N)	Cohesiveness
10/30	14.5 (1.01)[ab]	0.18 (0.023)[b]	45.1 (1.19)[a]	2.0 (0.04)[c]
15/25	16.9 (1.75)[a]	0.21 (0.035)[a]	56.9 (1.52)[b]	2.2 (0.06)[b]
20/20	17.9 (1.16)[b]	0.19 (0.055)[b]	71.2 (2.80)[c]	2.0 (0.03)[c]
30/10	15.2 (0.59)[ab]	0.17 (0.033)[b]	80.8 (2.40)[d]	2.8 (0.07)[a]

[ab] Means within a column followed by a different superscript are different ($P < 0.05$). Standard errors are in parentheses.

Table 4—Kramer shear peak force means and standard errors for beef frankfurters chopped at different temperatures

Chopping temperature (°C)	Peak force (N)
9	0.17 (0.037)[b]
12	0.21 (0.043)[a]
15	0.18 (0.037)[b]

[ab] Means within a column followed by a different superscript are different ($P < 0.05$). Standard errors are in parentheses.

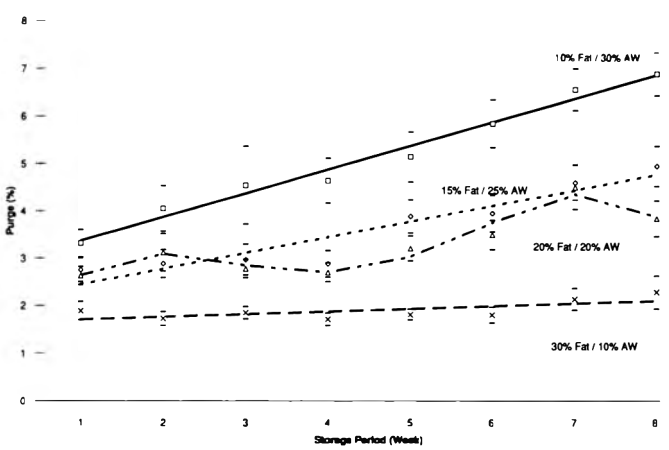


Fig. 2—Frankfurter percentage purge as influenced by formulation and storage period. Lines are the treatment regression lines. Symbols are treatment means with standard errors.

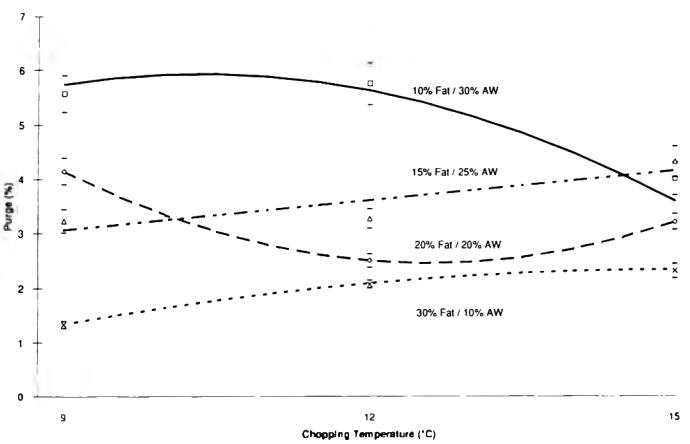


Fig. 3—Frankfurter percentage purge as influenced by formulation and chopping temperature. Lines are the treatment regression lines. Symbols are treatment means with standard errors.

Sodium Lactate/Sodium Chloride Effects on Aerobic Plate Counts and Color of Aerobically Packaged Ground Pork

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ABSTRACT

Ground pork (15% fat) was formulated to contain 0% to 3.0% sodium chloride and/or 0% to 3.0% sodium lactate (SL) resulting in 28 treatment combinations. Five replications consisted of two with low initial microbial loads ($< 10^3$ CFU/g) and three with high initial loads ($> 10^5$ CFU/g). Ground pork (454g) was overwrapped in PVC and stored (4°C) aerobically for 21 d. A significant salt × SL interaction existed for redness of samples with low initial aerobic plate count (APC). Red color was best preserved by 2.0% or 3.0% SL. Initial pH values of low and high initial APC samples were similar. Initial product pH was best maintained by higher SL levels in low initial load samples and by higher sodium chloride levels in high initial load samples. Samples containing 3.0% SL had the lowest APC regardless of salt level.

Key Words: ground pork, lactate sodium chloride, aerobic plate counts, microbes

INTRODUCTION

MICROBIAL GROWTH IN FRESH MEAT is the primary factor associated with meat quality reduction, spoilage, and economic loss. Extended shelf-life and meat product safety require maintaining low microbial numbers during fabrication, packaging, and storage of meat at refrigeration temperatures. A variety of additives which have the potential for inhibiting microorganisms associated with fresh aerobically and vacuum-packaged meat products have been investigated.

The bacteriostatic properties of lactic acid are well-documented (Chen and Shelef, 1992); however, concentrations > 1.25% resulted in unacceptable lean tissue discoloration and concentrations > 2.0% resulted in sour flavors (Mendonca et al., 1989; Woolthuis and Smulders, 1985). Alterations in pH can affect meat product color. This is the major determinant of consumer perceptions regarding meat product quality.

Sodium lactate (SL) is approved for use as a flavor enhancer (CFR 184.1768). Several research groups (Bradford et al., 1993; Brewer et al., 1991, 1992, 1993; Egbert et al., 1992; Lamkey et al., 1991; Papadopoulos et al., 1991a, b; Stillmunkes et al., 1993) have reported the effects of sodium or potassium lactate (SL or PL) on microbial growth in refrigerated meat systems. SL resulted in fewer color and flavor problems than lactic acid. Lamkey et al. (1991) reported that SL was effective in maintaining low microbial numbers for extended times in fresh pork sausage chubs stored at 4°C. We reported that 2.0% or 3.0% SL inhibited development of off-flavor in aerobically packaged fresh ground pork sausage and that standard aerobic plate counts (APC) during refrigerated storage were 10^1 to 10^2 CFU/g lower than controls (Brewer et al., 1991). Addition of 1.0% to 2.0% SL to vacuum-packaged fresh pork sausage increased shelf-life by a minimum of 2 wks, extended lag phase of aerobic bacteria, altered growth rate of anaerobic bacteria, and inhibited growth of anaerobic lactic acid-producing bacteria (Brewer et al., 1993).

Our objective was to examine the effects of sodium lactate and sodium chloride on microbiological quality and color of aerobically packaged ground pork with high vs low initial APC in refrigerated storage.

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MATERIALS & METHODS

Ground sample preparation

Meat batches (5 batches, 30 kg/batch) were prepared from lean (90% lean) and fat (50% lean) pork trimmings obtained 24 hr postmortem from sow carcasses at the University of Illinois abattoir. Trimmings were ground separately through a 2.5 cm plate, and fat content of each fraction was determined (AOAC, 1980). Lean and fat were combined to yield 15 ± 1% total fat content, then reground through a 0.3 cm plate. Each batch (30 kg) was subdivided into 1 kg batches. Sodium chloride (0%, 0.5%, 1.0%, 1.5%, 2.0%, 2.5%, or 3.0%) and SL (0%, 1.0%, 2.0%, or 3.0%, on a dry weight basis) were mixed into ground pork for 2 min using a KitchenAid model K5-A mixer (Hobart Mfg. Co., Troy, OH) with the paddle attachment at speed 2. Equipment and raw materials were chilled to 4°C prior to mixing. Ground pork (100 g) was placed on styrofoam trays, overwrapped with commercial PVC film (RMF-61, Borden, Chicago, IL; O_2 transmission rate = 5000 cc × 24 hr), and held at 4 ± 1°C for 21 days. Salt (Sigma Chem. Co., St. Louis, MO) and sodium lactate (SL-75, Trumark Inc., Linden, NJ; 60% solution) were reagent grade.

Microbiological analyses

Samples were prepared by pummelling three separate 10-g aliquots of meat from three locations in the package and 90 mL sterile 0.1% peptone for 45 sec in a stomacher (Tekmar Co., Cincinnati, OH). Subsequent dilutions were made in 0.1% sterile peptone water. Aerobic plate count was determined by spread plating 0.1 mL of sample homogenate. Each dilution was spread plated in triplicate for enumeration of aerobes on pre-poured Bacto Plate Count Agar (Difco Laboratories, Detroit, MI). Plates were incubated at 35°C aerobically for 48 hr before counting (Messer et al., 1984). Aerobic plate counts (APC) are the $\log_{10}$ of the number of colony forming units/g.

Color

Instrumental color determinations were made on the surface of chilled (4°C), packaged ground meat samples at each storage time immediately prior to microbiological sampling. Spectral reflectance was determined every 10 nm over the visible range (400–700 nm) under illuminant A at four locations on the meat surface using a HunterLab LabScan Spectrocolorimeter (model 6000, Hunter and Assoc., Reston, VA). CIE Lab values and hue angles (Tan^{-1} b*/a*) were calculated (CIE, 1978). L* (lightness), a* (redness), b* (yellowness), and hue angle (departure, in degrees, from a fixed red-green axis) were recorded. The difference between percent of light reflected at 630 nm and 580 nm was used as a measure of red color contributed by oxymyoglobin (Strange et al., 1974).

pH

Ten grams of sample were homogenized with 25 mL distilled water and pH was determined using a Fisher Accumet (model 830, Fisher Scientific, Itasca, IL) digital pH meter standardized at pH 4 and 7.

Statistical analyses

One master batch (30 kg) was formulated for each replication then subdivided into SL (0%, 1.0%, 2.0%, or 3.0%)/NaCl (0%, 0.5%, 1.0%, 1.5%, 2.0%, 2.5%, or 3.0%) groups (1 kg/group, 28 treatment combination groups). Five complete replications were conducted. Data from

Table 1—Effect of refrigerated storage time (pooled over sodium lactate and sodium chloride level) on selected characteristics of ground pork with low and high initial aerobic plate count (APC)

Characteristic	Time, days					
	0	7	11	14	18	21
Low init. APC[z]						
L* value[y]	55.26±.41^{bc}	56.36±.41^{b}	54.99±.41^{c}	55.30±.47^{bc}	58.61±.69^{a}	58.10±.69^{a}
a* value[x]*	21.20±.24^{a}	16.83±.24^{b}	14.12±.24^{c}	14.37±.27^{c}	13.58±.40^{c}	13.65±.40^{c}
b* value[w]	15.88±.21^{ab}	15.59±.21^{b}	15.10±.21^{b}	15.44±.23^{b}	16.36±.35^{a}	16.89±.35^{a}
% R diff[v]*	26.32±.43^{a}	16.76±.43^{b}	11.81±.43^{c}	12.34±.48^{c}	11.40±.72^{c}	11.97±.72^{c}
pH	5.87±.02^{c}	5.91±.02^{c}	5.98±.02^{b}	6.03±.03^{ab}	6.16±.04^{a}	6.12±.04^{a}
APC[u]*	2.93±.13^{e}	3.99±.13^{d}	4.80±.13^{c}	5.39±.18^{b}	4.99±.22^{bc}	6.81±.22^{a}
High init. APC[t]						
L* value[y]	54.74±.42	54.96±.43	56.02±.55	55.56±.83	—[s]	—
a* value[x]*	20.24±.25^{a}	14.82±.25^{b}	14.05±.32^{b}	14.29±.49^{b}	—	—
b* value[w]	17.24±.24^{a}	15.38±.25^{c}	16.46±.32^{ab}	15.94±.48^{bc}	—	—
% R diff[v]*	23.04±.49^{a}	13.16±.50^{b}	11.97±.63^{b}	11.99±.97^{b}	—	—
pH	5.80±.04^{d}	6.06±.04^{c}	6.25±.05^{b}	6.64±.09^{a}	—	—
APC[u]	4.72±.21^{c}	6.32±.21^{b}	5.87±.26^{b}	7.19±.44^{a}	—	—

[abcde] Means in a row with different superscript letters are different ($p < 0.05$).
[z] Low (< 10^3 CFU/g) initial aerobic plate count samples; number/Least Squares mean = 56.
[y] L* = lightness.
[x] a* = redness.
[w] b* = yellowness.
[v] Percent reflectance at 630 nm minus that at 580 nm.
[u] APC = $\log_{10}$ colony forming units/g.
[t] High (> 10^5 CFU/g) initial aerobic plate count samples; n/LS mean = 84.
[s] Data not collected at these times.
* Significant ($p < 0.05$) interactions existed for this characteristic.

this study were divided into replications with "low" initial microbial load (< 10^3 CFU/g) and replications with "high" initial microbial load (> 10^5 CFU/g). Data from replications with "high" initial loads (three replications) were pooled as were data from replications with "low" initial loads (two replications). Data were then handled as four (SL levels) × seven (salt levels) × four (storage times) for "high" initial load or 6 (storage times) for "low" initial load factorial designs and were subjected to Analysis of Variance using the General Linear Model (SAS Institute, Inc., 1988). Least Squares means and probability of difference were calculated for significant ($p < 0.05$) main effects and interactions (SAS Institute, Inc., 1988).

RESULTS & DISCUSSION

NO SIGNIFICANT ($p > 0.05$) three-way (SL × salt × storage time) interactions existed within either high or low initial load groups for any characteristic studied; therefore, only two-way interactions and main effects are presented.

Color

No significant two-way interactions existed for L* value. Ground pork with low initial APC became lighter (higher L* value) over time in refrigerated storage (Table 1). Storage time had no effect ($p > 0.05$) on lightness of pork with high initial APC (Table 1); however, it was not stored as long as low initial APC samples due to microbiological deterioration. Low initial APC samples containing 2.0% SL were slightly lighter than those containing 0%, 1.0%, or 3.0% SL; however, SL had no effect ($p > 0.05$) on lightness of samples with high initial APC (Table 2). The lack of SL effect on L* value supported our previous findings with fresh pork sausage with initial APC of 10^6 CFU/g (Brewer et al., 1991), and those of Egbert et al. (1992) with low fat ground beef patties containing carrageenan and potassium lactate. In our present study, salt had no effect ($p > 0.05$) on sample lightness (Table 3).

A significant SL by salt interaction was found for redness (a* value) (Fig. 1) and red color due to oxymyoglobin (% R diff) (data not shown) for samples with low initial APC. Control samples (0% SL, 0% salt) were reddest (highest a* value and % R diff). As salt concentration increased, redness of 0% SL samples decreased. Rhee et al. (1983) reported that in raw ground pork samples a* values were lowest and lipid oxidation (TBA values) was highest for samples containing NaCl; after 30 days storage, salt-containing samples had a pronounced greenish-brown color.

Table 2—Effect of sodium lactate (pooled over sodium chloride level and storage time) on selected characteristics of refrigerated ground pork with low and high initial aerobic plate counts (APC)

Characteristic	Sodium lactate, %			
	0	1.0	2.0	3.0
Low init. APC[z]				
L* value[y]	55.67±.51^{b}	56.45±.43^{b}	57.64±.39^{a}	55.79±.39^{b}
a* value[x]*	16.45±.26	15.65±.25	15.42±.20	15.98±.20
b* value[w]	16.45±.26^{a}	15.65±.22^{b}	15.42±.20^{b}	15.98±.20^{ab}
% R diff[v]*	14.63±.53	14.47±.44	15.75±.41	15.35±.40
pH*	5.98±.03	5.99±.03	6.03±.02	6.03±.02
APC[u]*	5.62±.17^{a}	5.26±.14^{b}	4.49±.13^{c}	3.71±.13^{d}
High init. APC				
L* value[y]	54.64±.70	55.02±.61	55.34±.45	55.56±.83
a* value[x]*	17.35±.42^{a}	15.71±.36^{b}	15.73±.27^{b}	14.60±.25^{c}
b* value[w]	16.48±.41	15.79±.36	16.35±.26	16.41±.24
% R diff[v]*	17.77±.82^{a}	15.47±.71^{bc}	14.09±.53^{cd}	12.84±.49^{d}
pH	6.27±.08^{a}	6.31±.06^{a}	6.15±.04^{a}	6.02±.04^{b}
APC[u]	6.83±.37^{a}	6.15±.30^{ab}	5.90±.22^{b}	5.23±.21^{c}

[abcd] Means in a row with different superscript letters are different ($p < 0.05$).
[z] Low (< 10^3 CFU/g) or high (> 10^5 CFU/g) initial aerobic plate count samples; low APC means are averaged over 21 d; high APC means are averaged over 14 days.
[y] L* = lightness.
[x] a* = redness.
[w] b* = yellowness.
[v] Percent reflectance at 630 nm minus that at 580 nm.
[u] APC = $\log_{10}$ colony forming units/g.
* Significant ($p < 0.05$) interactions existed for this characteristic.

Redness reduction may be the result of sodium-chloride promoted oxidation (Rhee, 1988). Samples containing 2.0% or 3.0% SL were redder than those containing 0% or 1.0% SL in the 1.0% to 2.0% salt range. Samples containing SL were redder than controls at 3.0% salt. No SL by salt interaction existed for red color of high initial APC samples. Initial (day 0) redness (a* value) was highest for 3.0% SL and lowest for 1.0% SL (Fig. 2). After 7 d and throughout the remainder of storage, 3.0% SL-containing samples were least red while 0% SL-containing samples were most red. A similar trend occurred for red color due to oxymyoglobin (data not shown). Low initial APC samples had similar a* values on day 0 regardless of salt concentration; however, after 14 d in refrigerated storage, samples containing 2.5% and 3.0% salt were less red than other samples (Fig. 3). A similar trend occurred in high initial APC samples starting after 7 d in refrigerated storage (data not shown).

No significant two- or three-way interactions occurred for yellowness (b* value) of samples with either low or high initial

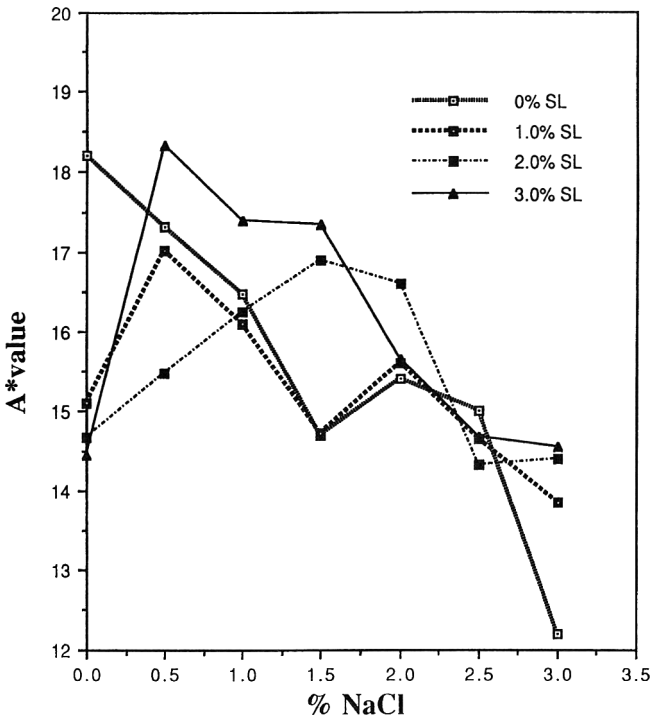


Fig. 1—Sodium lactate (SL)/salt effects on a* value of pork with low initial aerobic plate count. Standard error of the mean = 0.77. Data are pooled over storage time.

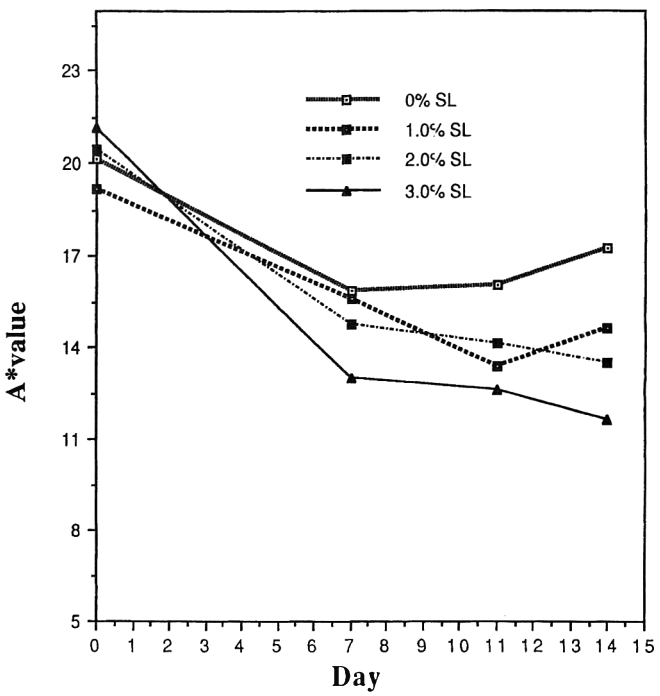


Fig. 2—Sodium lactate (SL)/storage time effects on a* value of pork with high initial aerobic plate count. Standard error of the mean = 0.42. Data are pooled over salt level.

Table 3—Effect of salt on selected characteristics of refrigerated ground pork with low and high initial aerobic plate counts (APC)

	Salt, %						
Characteristic	0	0.5	1.0	1.5	2.0	2.5	3.0
Low init. APC[z]							
L* value[y]	56.87 ± .71	56.29 ± .59	56.58 ± .60	56.35 ± .36	56.81 ± .52	55.88 ± .52	56.46 ± .52
a* value[x]*	15.61 ± .40^{b}	17.04 ± .39^{a}	16.56 ± .34^{b}	15.92 ± .30^{b}	15.82 ± .29^{b}	14.67 ± .30^{c}	13.75 ± .29^{d}
b* value[w]	15.62 ± .35	16.49 ± .30	16.28 ± .30	15.82 ± .26	15.89 ± .26	15.59 ± .27	15.44 ± .26
% R diff[v]*	16.41 ± .73^{abc}	18.05 ± .61^{a}	16.79 ± .62^{ab}	15.40 ± .54^{bc}	15.11 ± .53^{c}	15.40 ± .54^{bc}	11.84 ± .53^{d}
pH*	6.26 ± .04^{a}	6.12 ± .03^{b}	5.99 ± .04^{c}	5.93 ± .03^{c}	5.94 ± .03^{c}	5.93 ± .03^{c}	5.93 ± .03^{c}
APC[u]*	5.64 ± .23^{a}	5.53 ± .19^{a}	5.33 ± .20^{a}	4.68 ± .18^{b}	4.43 ± .17^{b}	4.02 ± .17^{c}	4.10 ± .17^{c}
High init. APC[z]							
L* value[y]	55.74 ± .80	55.07 ± .78	55.21 ± .77	55.44 ± .77	55.15 ± .65	55.99 ± .57	55.64 ± .58
a* value[x]*	16.82 ± .47^{a}	16.76 ± .46^{a}	16.34 ± .46^{a}	15.63 ± .46^{b}	15.79 ± .39^{b}	15.19 ± .34^{b}	14.41 ± .34^{c}
b* value[w]	15.60 ± .46	16.28 ± .45	15.34 ± .44	15.87 ± .44	16.48 ± .38	16.45 ± .33	16.78 ± .34
% R diff[v]*	18.57 ± .93^{a}	17.57 ± .90^{a}	16.23 ± .89de	13.82 ± .89^{b}	13.44 ± .76^{b}	13.51 ± .66^{b}	12.15 ± .68^{b}
pH	6.38 ± .08^{a}	6.31 ± .08^{ab}	6.26 ± .08^{ab}	6.23 ± .08^{abc}	6.13 ± .07^{bc}	6.06 ± .06^{cd}	5.93 ± .06^{d}
APC[u]	6.72 ± .42	6.26 ± .39	6.36 ± .34	6.10 ± .38	5.81 ± .33	5.61 ± .28	5.32 ± .28

^{abcde} Means in a row with different superscript letters are different ($p < 0.05$).
[z] Low (< 10^3 CFU/g) or high (> 10^5 CFU/g) initial aerobic plate count samples; low APC means are averaged over 21 d; high APC means are averaged over 14 days.
[x] L* = lightness.
[y] a* = redness.
[w] b* = yellowness.
[v] Percent reflectance at 630 nm minus that at 580 nm.
[u] APC = $\log_{10}$ colony forming units/g.
* Significant ($p < 0.05$) interactions existed for this characteristic.

APC. Storage time and SL had inconsistent effects on b* value (Tables 1 and 2).

APC and pH

Initial pH of ground pork was unaffected by SL addition (data not shown). Lamkey et al. (1991) and Brewer et al. (1991, 1992, 1993) also reported no SL effects on initial product pH. Pork with low initial APC had an initial pH (day 0) of 5.77. A significant SL by salt interaction existed for pH of ground pork with low initial APC (Fig. 4). In pork containing 0% or 0.5% NaCl, samples with 0% or 1.0% SL had higher pH than those with 2.0% or 3.0% SL. However, in pork with 1.5% to 3.0% salt, samples with 0% or 1.0% SL had lower pH than those with 2.0% or 3.0% SL.

The initial (day 0) pH of pork with high initial APC was 5.75. No significant two- or three-way interactions existed for pH in high APC samples. Over time, pH increased (Table 1). Samples with 3.0% SL had lower pH than those with 0%, 1.0%, or 2.0% SL (Table 2). Samples containing 2.5% or 3.0% salt had the lowest pH, while those containing 0% to 1.5% salt had the highest pH.

In general, addition of SL has not had significant effects on initial pH of meat products. However, with time in refrigerated storage, SL sometimes reduced pH decline probably by limiting growth of acid-producing bacteria, particularly in vacuum-packaged products (Brewer et al., 1991, 1992, 1993; Lamkey et al., 1991; Maas et al., 1989). Two mechanisms have been proposed for observed SL and PL suppression of microbes. These salts may lower water activity (Chen and Shelef, 1992) or they may cross the cell membrane, dissociate, and acidify the interior of

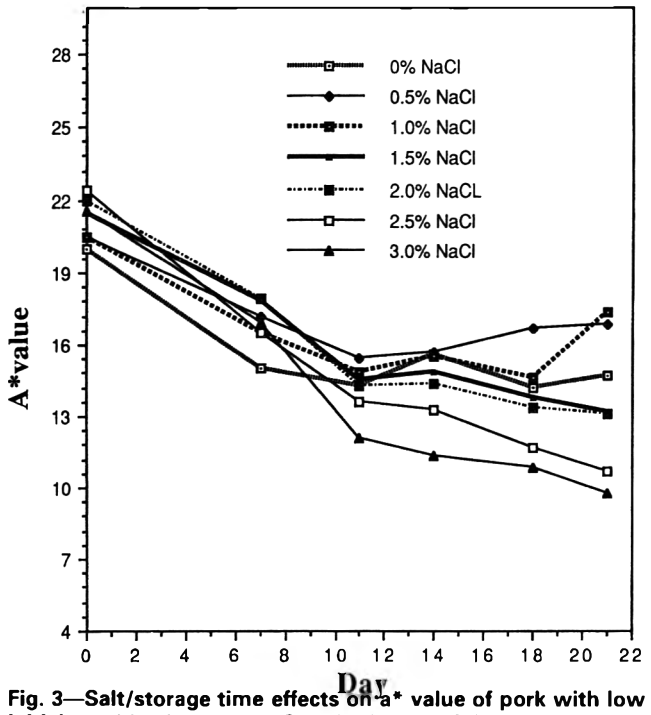


Fig. 3—Salt/storage time effects on a* value of pork with low initial aerobic plate count. Standard error of the mean = 0.40. Data are pooled over sodium lactate level.

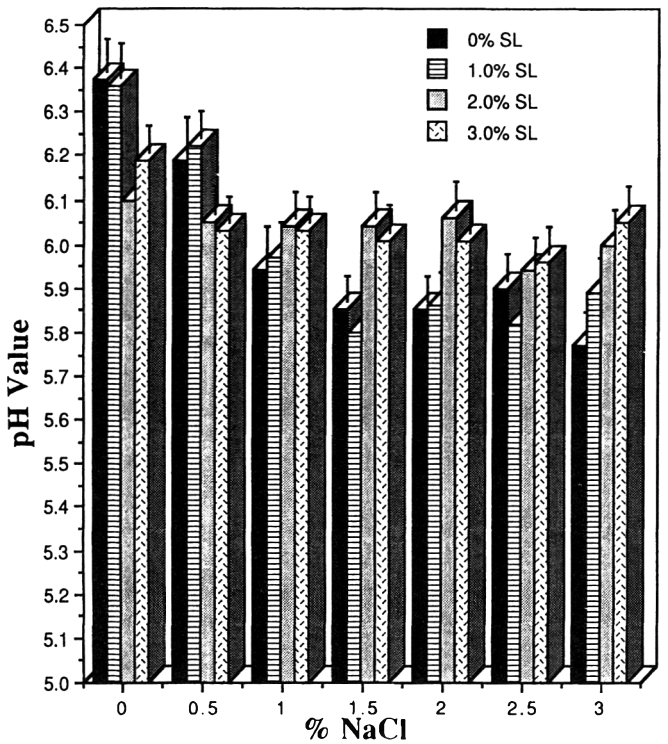


Fig. 4—Sodium lactate (SL)/salt effects on pH of pork with low initial APC. Standard error of the mean = 0.34. Data are pooled over storage time.

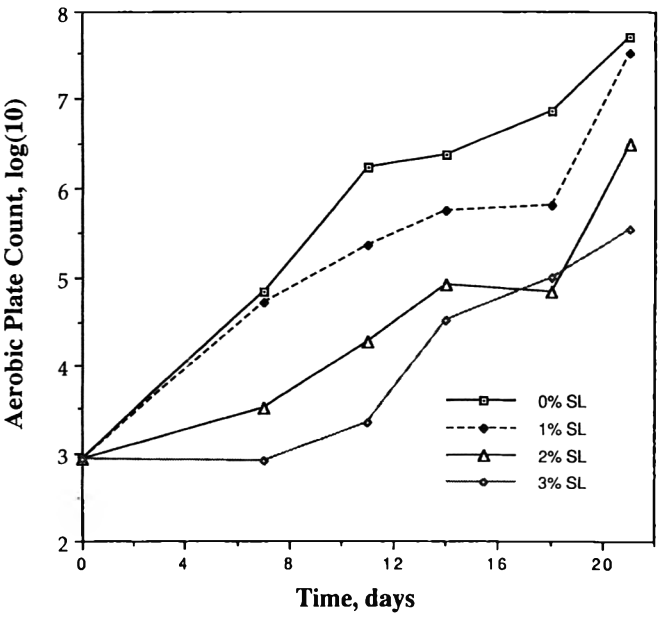


Fig. 5—Sodium lactate (SL)/storage time effects on aerobic plate count (APC) of pork with low initial APC. Standard error of the mean = 0.30. Data are pooled over salt level.

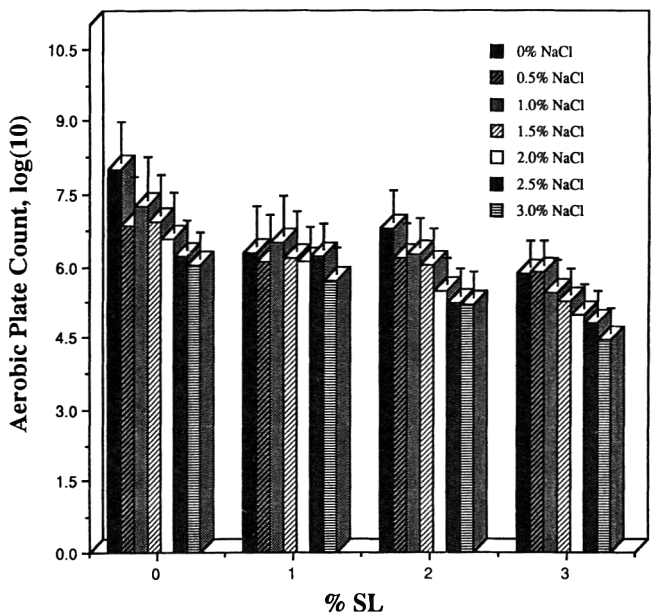


Fig. 6—Sodium lactate (SL)/salt effects on aerobic plate count (APC) of pork with high initial APC. Standard error of the mean = 0.81. Data are pooled over storage time.

the cell (Conner et al., 1990; Maas et al., 1989). Egbert et al. (1992) reported that pH of carrageenan-based, low fat beef patties containing 2.0% or 3.0% potassium lactate did not change during refrigerated storage while pH of patties containing 0% or 1.0% lactate increased. Nassos et al. (1985) reported similar pH increases during refrigerated storage of ground beef. They indicated that beef samples with higher pH had larger populations of Gram-negative bacteria. The dominant Gram-negative bacteria associated with fresh red meats with higher pH ($\geq$ 5.6) have been the *Pseudomonas* spp., a proteolytic group capable of increasing product pH (Ahmad and Marchello, 1989; Banwart, 1981). Nassos et al. (1985) reported that lactic acid concentrations in refrigerated beef correlated with proportions of Gram-positive bacteria (lactic acid bacteria); Gram-positive bacteria correlated negatively with pH. Although we made no attempts to identify bacteria it is likely that SL suppressed distinctly different bacterial groups than did salt. Different degrees of suppression of acid-producing bacteria in relation to putrefactive bacteria capable of releasing free ammonia may be responsible for our observed pH changes.

No significant three-way interactions existed for APC of low initial APC samples. When data were pooled over time, samples containing 3.0% SL had the lowest APC ($\approx 10^4$ CFU/g) at each salt concentration. Salt $\geq$ 1.5% was required to reduce mean APC in samples containing no SL (data not shown). When low initial APC data were pooled over salt level, those containing no SL were spoiled (10^7 CFU/g) after about 18 d of refrigerated storage. Those containing 2.0% or 3.0% SL had not reached spoilage levels at 21 d, and the lag phase of growth appeared

to be depressed (Fig. 5). Lamkey et al. (1991) and Brewer et al. (1992; 1993) reported similar effects on lag phase of total aerobes. Shelef and Yang (1991) reported that 4% to 5% SL extended lag phase of three strains of *Listeria monocytogenes* by 1 to 2 wk at 5°C. SL and salt had less notable effects on APC of samples with high initial microbial loads in our present study (Fig. 6). In samples containing no SL, salt decreased APC. SL was less effective in suppressing microbial growth than was salt in samples with high initial APC.

CONCLUSIONS

RED COLOR (a* value, % reflectance difference) was best in low initial microbial load pork containing 3% SL and 0.5% to 2.0% salt. APC was best controlled by 3% SL and no salt; with 2% SL, salt concentrations of 1.5% to 2.0% were required for maximum APC suppression. If no SL were added, 3% salt was required for maximum APC reduction. Pork with high initial microbial load required near maximum levels of SL (2–3%) and salt (2–3%) to suppress APC; these levels had negative effects on red color. These results emphasize the importance of high sanitation standards during meat processing to maintain sensory and shelf-life characteristics of products.

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Variations in Radiation Sensitivity of Foodborne Pathogens Associated with the Suspending Meat

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ABSTRACT

Longissimus dorsi from beef, pork, and lamb and turkey breast and leg meats were inoculated with *Escherichia coli* O157:H7, *Listeria monocytogenes*, *Salmonella* spp., and *Staphylococcus aureus*, and the gamma radiation resistance of the pathogens were determined under identical conditions. At 5°C the respective radiation D-values of *E. coli* O157:H7 and *L. monocytogenes* did not vary with the suspending meat. The D-value for a mixture of *Salmonella* spp. was significantly lower on pork than on beef, lamb, turkey breast, and turkey leg meats. The D-value for *S. aureus* was significantly lower on lamb and mechanically deboned chicken meat than on the other meats. All values were, nevertheless, within expected ranges.

Key Words: pathogens, bacteria, radiation sensitivity

INTRODUCTION

SINCE BOTH THE COMPOSITION and the radiation chemistry of the major chemical components of beef, pork, lamb, and poultry meats are well known, it should be possible to predict changes that may occur when those food products are irradiated (Diehl, 1990; Josephson, 1983; Merritt and Taub, 1983; Taub, 1981). Diehl and Scherz (1975) and Taub et al. (1976) concluded that it is possible to assess wholesomeness with a reasonable degree of certainty by extrapolating chemical data from one food and applying it to another that is generically related. The concept of "chemiclearance" may be important in decisions about approval for irradiation of red meats and possibly other meats not included in existing regulations by the U.S. Food & Drug Administration and for which extensive toxicological data are scarce. The primary purpose for irradiating meats is to control or eliminate foodborne pathogens. Yet, there are many published examples of significantly different radiation D-values for a given pathogen on different meats under irradiation conditions that were apparently very similar (Urbain, 1986). Primary factors such as temperature during irradiation and presence or absence of oxygen can significantly alter the estimates of radiation sensitivity. Some secondary factors which may also have an influence are growth media and incubation conditions for propagation and estimation of pathogen populations following irradiation. In order to accept the concept of chemiclearance for toxicological data, regulatory agencies must have confidence that the efficacy of the process to control or eliminate foodborne pathogens is reasonably predictable between generically related products as well as between poultry and red meats.

Our objective was to test the "null hypothesis" that the radiation resistance of *Escherichia coli* O157:H7, *Salmonella spp.*, *Listeria monocytogenes*, and *Staphylococcus aureus* on different meats does not differ when irradiation and culture conditions are identical. A second parallel study tested the "null hypothesis" that chemical changes in meats irradiated under identical conditions are either the same or predictable from chemical composition or physical structure.

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MATERIALS & METHODS

Experimental design

The radiation resistance of two gram-positive and two gram-negative foodborne pathogens were studied simultaneously on beef, lamb, pork, turkey breast meat, and turkey leg meat. To avoid possible effects of culture variation, all five meats were inoculated from the same culture at the same time and irradiated under identical conditions at the same time. Because tissues are known to have animal-to-animal variations, three independent lots of each meat were obtained at different times, and the entire study was replicated.

Cultures

Three isolates of *Escherichia coli* O157:H7 35150, 43889, and 43894; *Salmonella dublin* 15480, *S. enteritidis* 13076, *S. newport* 6962, *S. senftenberg* 8400, *S. typhimurium* 14028; *Staphylococcus aureus* 25923, and 13565; and *Listeria monocytogenes*, 15313, 43256, 49594, and 7644 were obtained from the American Type Culture Collection, Rockville, MD. *E. coli* 93-937 was associated with a restaurant outbreak and was obtained from the Oregon Public Health Laboratory (Portland, OR), and ENT C9490 was associated with the major outbreak in Oregon during 1992 and was obtained from the Communicable Disease Center (Atlanta, GA). *S. aureus* B124 was obtained from the Eastern Regional Research Center culture collection. All cultures were maintained and cloned on Tryptic Soy Agar (TSA, Difco, Detroit, MI). Culture identity was confirmed by gram stains and from reactions on GNI or GPI cards, as appropriate, of the Vitek AMS Automicrobic System (bioMérieux Vitek, Inc., USA, Hazelwood, MO) (Aldridge et al., 1977; Knight et al., 1990). Each isolate was cultured independently in 100 mL of tryptic soy broth (TSB. Difco, Detroit, MI) at 35°C with agitation at 150 rpm on a rotary shaker for 18 hr at 35°C. Equal amounts of the culture of each isolate of a pathogen were mixed, and the culture was harvested by centrifugation. A tenfold inoculum was prepared by resuspending the cells in 1/10 volume of Butterfield's phosphate (0.25M KH_2PO_4 adjusted to pH 7.2 with NaOH).

Substrates and packaging conditions

All meats were purchased locally the day after slaughter. Three lots of each meat were obtained at different times. Pork was purchased from Leidy of Souderton, PA; beef (steer) from Carl Venezia of Conshohocken, PA; lamb, and turkey from C. Fehl's of Springhouse, PA. The Longissimus dorsi of the mammals and the breast and all of the leg muscles of the turkey were used. A single muscle was selected from the red meats to avoid introducing variances due to tissue. Turkey breast meat is relatively homogeneous, and virtually no information is available on the effects of radiation on foodborne pathogens on either breast or leg meats. Turkey also provides a comparison to data already available for the survival of these pathogens on chicken. All muscles were carefully trimmed of fat, cubed, and frozen in dry ice. The meat was then pulverized in a Hobart silent cutter to yield a homogeneous material. Mechanically deboned chicken meat (MDCM) consisting of ≈ 90% rib and 10% back meat was obtained in 18-kg lots from a commercial manufacturer of poultry frankfurters, Tyson Foods, New Holland, PA. The meat substrates were subdivided into 100 ± 0.05g amounts and then spread thinly and vacuum sealed in Stomacher 400 (Tekmar Co., Cincinnati, OH) polyethylene bags. These bags were then vacuum sealed in high barrier pouches fabricated with 0.025 mm polycaprolactam (nylon 6) as the outside layer, 0.0090 mm aluminum foil as the middle layer, and 0.051 mm polyethylene terephthalate as the inner layer (American National Can Company, Des Moines, IA) to provide better protection during handling and to prevent oxygen transmission to the samples. The meat was frozen at −50°C and sterilized by gamma irradiation to a dose of 42 kGy at −30°C. Prior research (Thayer et al., 1987; Thayer, 1990;

Table 1—Effect of suspending meat on gamma radiation D-values at 5°C for foodborne pathogens *E. coli* O157:H7, *L. monocytogenes, Salmonella spp.,* and *S. aureus*

Pathogen	Beef D(kGy) ± SE	Lamb D(kGy) ± SE	Pork D(kGy) ± SE	Turkey breast D(kGy) ± SE	Turkey leg D(kGy) ± SE	MDCM D(kGy) ± SE
E. coli O157:H7	0.30 ± 0.02[a]	0.32 ± 0.02[a]	0.30 ± 0.01[a]	0.30 ± 0.01[a]	0.29 ± 0.04[a]	ND
L. monocytogenes	0.45 ± 0.03[b]	0.47 ± 0.04[b]	0.48 ± 0.02[b]	0.50 ± 0.03[b]	0.47 ± 0.03[b]	ND
Salmonella spp.	0.70 ± 0.04[c]	0.67 ± 0.04[c]	0.51 ± 0.03[d]	0.71 ± 0.04[c]	0.71 ± 0.04[c]	ND
S. aureus	0.46 ± 0.02[e]	0.40 ± 0.03[f]	0.43 ± 0.02[e]	0.45 ± 0.03[e]	0.46 ± 0.05[e]	0.41 ± 0.03[f]

[a,b,c,d,e,f] Different letters in the same row indicate significant differences ($P < 0.05$); MDCM = mechanically deboned chicken meat; SE = standard error; ND = not determined; *Salmonella spp.* = *S. dublin, S. enteritidis, S. newport, S. senftenberg,* and *S. typhimurium.*

Thayer and Boyd, 1991) demonstrated that such treatments did not significantly alter the wholesomeness and nutritional characteristics or the response of *Salmonella typhimurium* on the meat to gamma radiation. Both sterile and non-sterile meat were stored at −50°C until use.

Radiation source and irradiation techniques

The self-contained gamma-radiation source of ^{137}Cs had a strength of approximately 134,000 Ci (4.95 PBq) and a dose rate of 0.108 kGy min^{-1}. The dose rate was established using National Physical Laboratory (Middlesex, United Kingdom) dosimeters. Variations in doses absorbed by experimental samples were minimized by placement within a uniform area of the radiation field. Samples were maintained at 5 ± 0.5°C during irradiation by injecting the gas phase from liquid nitrogen into the irradiation chamber. Sample temperature was monitored continuously during irradiation.

Inoculation of meat for determination of D_{10} values

Sterile meat was thawed rapidly in about 5 min by submerging the package in a 50°C water bath, inoculated with sufficient cells for a final population of ≈ 10^9 stationary-phase cells/g, and mixed in sterile Number 400 polyethylene Stomacher bags by stomaching for 90 sec using a Stomacher 400 (Tekmar Co., Cincinnati, OH). Aliquots of 5.0 ± 0.05g of inoculated meat were transferred aseptically to radiation-sterilized oxygen permeable poultry bags (E-300, Cryovac Division, W. R. Grace & Co., Duncan, SC). These bags complied with U.S. regulations. Inoculated meat was spread uniformly over an area of about 10 × 10 cm within the bags and heat sealed in vacuo.

Determination of D_{10} values

Inoculated meat samples received radiation doses of 0 to 2.0 kGy in increments of 0.235 kGy at 5 ± 0.5°C. The studies of *E. coli* O157:H7, *L. monocytogenes*, and *Salmonella* were repeated three times. The study of *S. aureus* was repeated twice.

Microbiological analysis

Samples were assayed for colony-forming units (CFU) by standard pour-plate procedures using TSA with serial dilutions in sterile Butterfield's phosphate. Petri plates containing *E. coli* O157:H7, *Salmonella spp.* or *S. aureus* were incubated for 24 hr at 35°C before counting. Petri plates containing *L. monocytogenes* were incubated for 48 hr at 35°C before counting. CFU were counted on three petri plates having 30 to 300 colonies with a New Brunswick Scientific Biotran II® automated colony counter (New Brunswick Scientific Co., Inc., Edison, NJ).

Proximate analysis

Water and fat were determined by CEM methods (AOAC, 1990) with one modification: After determining moisture content, the samples for fat determination were blended with 100 mL methylene chloride, the slurry was quantitatively transferred to filter paper in a Buchner funnel, and the methylene chloride removed by suction. Protein and ash were determined using these CEM microwave furnaces; MAS-300 furnace for ash (CEM, 1990; Zheng and Dotson, 1994) and Kjel-FAST furnace for Kjeldahl digestion (CEM, 1987). After sulfuric acid/H_2O_2 digestion in the CEM oven, the alkalization and distillation of the ammonia was completed in a Kjeltec apparatus (AOAC, 1990).

Statistical analysis

Cultural responses were expressed as the logarithm of the CFU/g. For each experiment, the average (N) CFU value of 3 plate counts for each replicate sample was determined and divided by the average of the 3 zero-dose values (N_o) to give a survivor value (N/N_o). The $\log_{10}$ survivor values ($\log_{10}(N/N_o)$) were then used for subsequent calculations. The D-values (dose in kGy resulting in a 90% reduction of viable CFU) were the reciprocals of the slopes of the linear regressions of the log survivor values as determined by least squares analysis. The zero-dose values were excluded from the calculation of the regression to avoid shoulder effects as described by Thayer et al. (1990). Statistical calculations were performed with the general linear models procedure of the SAS statistical package (Freund et al., 1986; SAS Institute, Inc. 1987). The regressions were tested for differences by analysis of covariance.

RESULTS

Radiation D_{10} values

The D-values for the four pathogens on beef, lamb, pork, turkey breast, and turkey leg were compared (Table 1). The D-value for *S. aureus* was greater than expected, based on our previous studies with MDCM (Thayer and Boyd, 1992). Therefore an additional set of three samples of MDCM was inoculated with the mixture of *S. aureus* isolates and the D-value for each replicate was determined.

The D-values for *E. coli* O157:H7 and *L. monocytogenes* were not significantly ($P < 0.05$) different on any of the 5 substrates tested (Table 1). The D-values for *Salmonella* on beef, lamb, turkey breast, and turkey leg meats were not different (Fig. 1). The D-value for *Salmonella* was lower ($P < 0.05$) on pork meat than on any of the other meats (Table 1, Fig. 1). The D-value for *S. aureus* on lamb or chicken meat was lower ($P < 0.05$) than when these microorganisms were on the other meats. The D-value on turkey leg meat was different from that on chicken meat (Table 1, Fig. 2).

Proximate analysis

The proximate analyses of the beef, lamb, MDCM, pork, turkey breast, and turkey leg meats were also compared (Table 2). The major differences between these products were in low and high fat contents in turkey breast meat and MDCM, respectively (Table 2).

DISCUSSION

WE KNOW OF NO PREVIOUS REPORTED STUDY in which radiation survival of foodborne pathogens was determined on 5 meat substrates under identical conditions. The "null hypothesis" that the suspending meat would not alter the radiation D-values for either *E. coli* O157:H7 or *L. monocytogenes* when the culture and irradiation conditions were identical was accepted. The null hypothesis was not accepted for the radiation survival of either *Salmonella* or *S. aureus*. We eliminated culture bias by inoculating meat samples from each of the four species at the same time using a single inoculum preparation. Animal bias was tested by performing three independent studies with different lots of meat from different animals for each of the studies. Thus, the differences in responses of *Salmonella* and *S. aureus* to

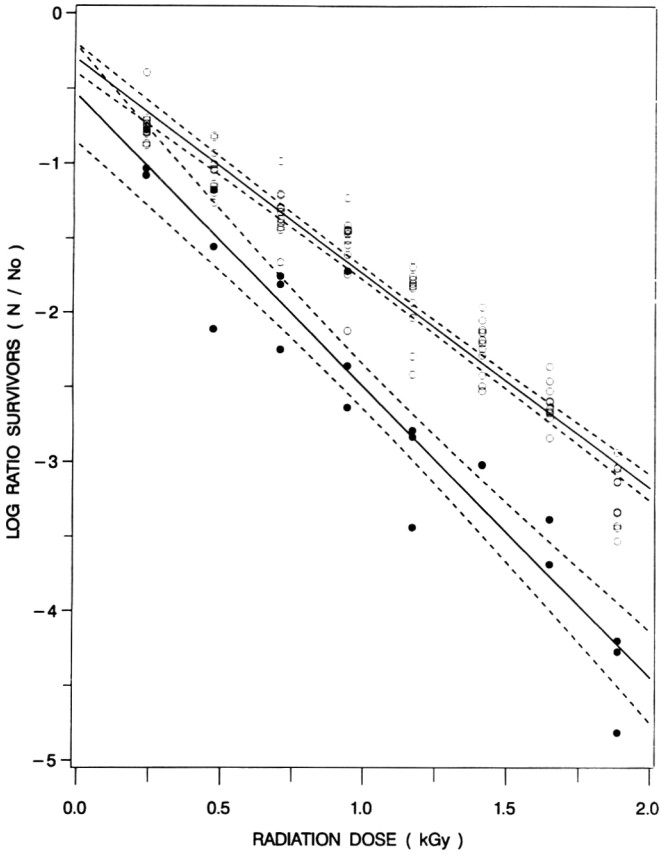


Fig. 1—Effect of gamma radiation on the survival of a mixture of *S. dublin, S. enteritidis, S. newport, S. senftenberg,* and *S. typhimurium* suspended on the L. dorsi of beef, pork, and lamb, turkey breast meat, and turkey leg meat. The linear regressions for the survival curves of the *Salmonella* on beef, lamb, turkey breast, and turkey leg meats are identical and are plotted as a single regression (○); the linear regression for the survival of *Salmonella* on pork (●) is plotted separately. The regression curves are shown with their 95% confidence limits as dashed lines.

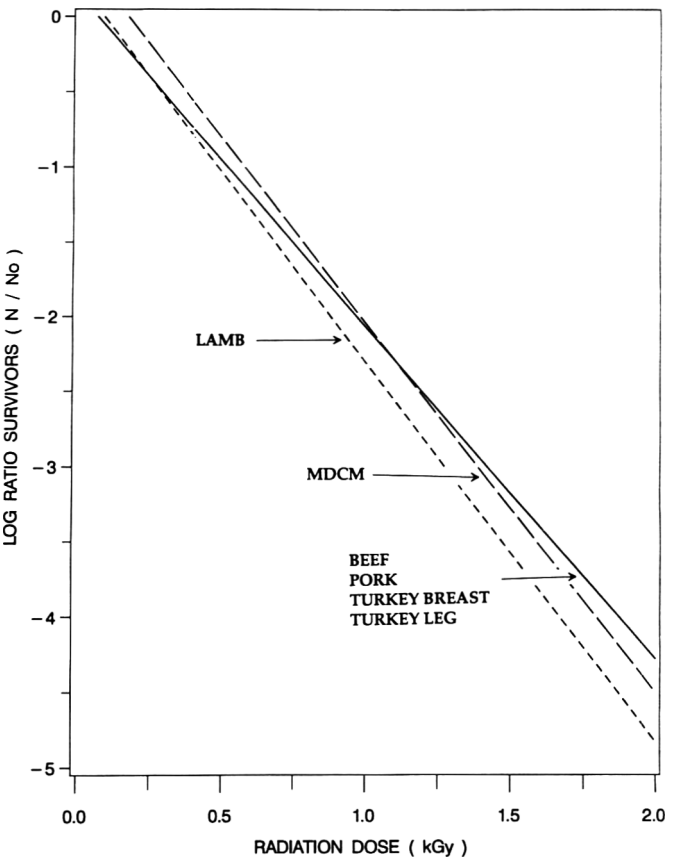


Fig. 2—Effect of gamma radiation on the survival of *S. aureus* on the L. dorsi of beef, pork, and lamb, turkey breast meat, turkey leg meat, and mechanically deboned chicken (MDCM). The survival curves for *S. aureus* on beef, pork, turkey breast, and turkey leg meats are identical and are presented as a single regression line. The survival curves for *S. aureus* on lamb, and MDCM are significantly different.

gamma radiation must be associated with chemical and/or physical properties of the meats. With exception of the MDCM, all meats were chopped using identical techniques, and the same muscle tissues were used for comparison of the red meats. So at the macro scale the physical state of each of the meats should have been very similar. The purpose of our companion study, mentioned earlier, was to examine these meats for any changes in chemical properties when irradiated.

Thayer and Boyd (1993) found a D-value of 0.28 kGy for *E. coli* O157:H7 irradiated in vacuo on finely ground lean beef at 5°C and a value of 0.27 kGy on either lean ground beef or MDCM. No effect of atmosphere during irradiation was found, nor was there an apparent effect of fat since the beef contained 2.6% and the MDCM contained 21.3% fat. Grant and Patterson (1991) obtained a D-value of 0.34 kGy for a nonpathogenic strain of *E. coli* irradiated in the presence of air on fresh pork fillets. Patterson (1988a) found D-values of 0.27 and 0.39 kGy for nonpathogenic *E. coli* irradiated at 10°C *in vacuo* or in air. The results of our present study were very similar to those reported previously, but they expand data for *E. coli* O157:H7 to include pork, lamb, and turkey.

Huhtanen et al. (1989) found a mean D-value of 0.43 kGy at 2-4°C for several isolates of *L. monocytogenes* on chicken meat. Patterson (1988b) found D-values of 0.417-0.553 kGy depending on strain and plating medium for *L. monocytogenes* on chicken mince. Beuchat et al. (1993) found D-values of 0.51 to 0.61 kGy for *L. monocytogenes* on ground beef irradiated at a commercial radiation source and did not find effects for either fat content or temperature on the results. In our study we found D-values of 0.45-0.50 kGy for *L. monocytogenes* irradiated at 5°C on beef, pork, and lamb L. dorsi muscle, turkey breast meat, and turkey leg meat. Individual values were not significantly different ($P > 0.05$) and are similar to those reported for chicken meat and hamburger. We could not find published radiation D-values of *L. monocytogenes* on pork, lamb, and turkey meats.

Many investigators examined effects of gamma radiation on *Salmonella* on various foods. Tarkowski et al. (1984) found D-values from 0.55 kGy for *S. typhimurium* to 0.78 kGy for *S. stanley* on raw ground beef irradiated at 18–20°C. Grant and Patterson (1991) determined the gamma radiation D-values for two strains of *S. typhimurium* irradiated on pork in air at 10°C to be 0.86 and 0.40 kGy. Thayer et al. (1990) determined the D-values for six species of *Salmonella* irradiated on MDCM at 4°C that in air ranged from 0.42 for *S. arizona* to 0.77 kGy for *S. enteritidis*. Patterson (1988a) found a D-value of 0.50 kGy for *S. typhimurium* irradiated in air on chicken mince at 10°C. No published radiation D-value determinations for *Salmonella* on turkey or lamb could be found. The radiation D-values we found for a mixture of five serovars were within the range of values reported previously on all of the meats and are identical with the exception of the values for pork. We could not account for the reduction in D-value on the mixture of *Salmonella* when irradiated on pork compared to D-values on the other meats. Values from proximate analysis of these products were very similar (Table 2). For example, the mean fat content of the pork samples was 3.07%, and the mean for all meats was 3.23%. Maxcy and Tiwari (1973) found D-values for *S. enteritidis* were 0.70 and 0.49 kGy in low- and high-fat beef, respectively. Beuchat et al. (1993), however, did not find a significant effect of

Table 2—Protein, fat, moisture, ash, and pH of meats

Meat	Protein % ± SD[a]	Fat % ± SD	Moisture % ± SD	Ash % ± SD	Total % ± SD	pH ± SD
Beef L. dorsi	19.4 ± 1.63	5.20 ± 1.34	70.8 ± 0.91	0.84 ± 0.07	96.3 ± 1.60	5.67 ± 0.10
Lamb L. dorsi	20.0 ± 1.83	3.85 ± 0.71	72.7 ± 1.12	0.96 ± 0.04	97.5 ± 1.51	5.53 ± 0.06
Pork L. dorsi	20.7 ± 2.01	3.08 ± 1.69	72.0 ± 0.30	1.08 ± 0.02	96.9 ± 0.75	5.39 ± 0.28
Turkey breast	23.4 ± 0.88	1.38 ± 0.73	72.6 ± 1.06	1.02 ± 0.08	98.5 ± 1.12	5.76 ± 0.13
Turkey leg	20.1 ± 0.96	2.65 ± 0.62	74.6 ± 0.65	0.97 ± 0.03	98.3 ± 0.83	6.03 ± 0.34
MDCM[a]	16.4 ± 5.59	21.9 ± 0.14	64.2 ± 0.14	0.97 ± 0.04	103.4 ± 5.34	6.71 ± 0.14

[a] SD = standard deviation; MDCM = mechanically deboned chicken meat.

fat content in beef on the radiation D-value for a mixture of five serovars of *Salmonella*.

The similarity of total fat content of the meat samples tends to eliminate fat level as a factor. If we examine the fatty acid composition of beef, pork, lamb, and turkey, the only major difference between these meats was that turkey had lower ratios of stearic and high ratios of linoleic acids than the other meats (Anderson, 1990, 1992; Posati, 1979). But the D-value for *Salmonella* was the same on both beef and turkey. We postulated that the difference may be due to a radiolytic product formed either during irradiation of the inoculated meat or possibly during the initial sterilization of the meat. The amounts of such radiolytic products, however, would be extremely small (ppb level, Merritt and Taub, 1983). Maxwell and Rady (1989) found only negligible changes in fatty acid profiles for neutral lipids of chicken muscle and for fatty acyl residues of skin lipids following irradiation in air at doses up to 10 kGy at 2–5°C. Thayer (1990) found no significant changes in fatty acids of radiation-sterilized chicken meat. If even relatively minor amounts of fatty acids such as linolenic acid were produced as radiolytic products we might have found some effect on *L. monocytogenes*, which is inhibitory at 100 µg/mL (Wang and Johnson, 1992). The pathogen cells were, however, not cultivated on the irradiated meats. Unless the reaction took place during irradiation any radiolytic products would be expected to be at non-toxic levels after dilution of the cultures for counting.

Thayer and Boyd (1992) obtained a D-value of 0.36 kGy for stationary phase cells of *S. aureus* ATCC 13565 irradiated at 0°C *in vacuo* on MDCM. Patterson (1988a) found D-values of 0.37 to 0.42 for a *S. aureus* isolate when irradiated in chicken mince under various atmospheres. Differences between D-values under different atmospheres were not considered significant. D-values of 0.58 and 0.30 kGy were found by Maxcy and Tiwari (1973) for the radiation resistance of *S. aureus* in low- and high-fat beef, respectively. However, Beuchat et al. (1993) did not find such an effect of fat content on radiation sensitivity of *S. aureus*. We found D-values from 0.40 kGy in lamb meat to 0.46 kGy in beef, pork, turkey breast, and turkey leg meats. Those values seemed higher than those obtained previously (Thayer and Boyd, 1992) with MDCM. We thus included a study with three replicate samples of MDCM and found a D-value of 0.41 kGy. As mentioned previously, results with lamb and MDCM were significantly different from those with beef. Nevertheless, the range of values was not great and was in reasonable agreement with the few published values available. Some reports indicate gamma radiation produces greater lethality for microorganisms at reduced pH. However, no significant difference in pH values of the meats were noted either before or after irradiation (Table 2). Differences in reductant values between skeletal muscle and liver, but not between skeletal muscles, were associated with differences in rates of radiolysis of thiamin by Fox et al. (1993).

We did not test for presence of antibiotics or other antimicrobial substances in the meats used to suspend the organisms. Possibly such substances could be present and interact with the test bacteria. However, this was considered unlikely since the meats were obtained from USDA inspected facilities. The samples were diluted immediately following irradiation and were not at any time incubated on the meat itself.

Extrapolations based on the higher radiation D-values for either *Salmonella* or *Staphylococcus* would provide greater protection for consumers. Because we could not explain changes in radiation D-values for *Salmonella* and *S. aureus* on the different meats, it seems prudent to not assume D-values for a pathogen would be the same on all meats. However, irradiation of non-frozen meats, based on current regulations for poultry, to a minimum dose of 1.5 kGy with a maximum dose of 3.0 kGy would greatly decrease any danger of *E. coli* O157:H7, *Listeria monocytogenes, Salmonella spp.* and *Staphylococcus aureus* reaching the consumer.

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Mention of a brand or firm name does not constitute an endorsement by the U.S. Department of Agriculture over others of a similar nature not mentioned.

*FORMULATION/CHOPPING TEMPERATURE FRANKFURTERS. . .*From page 57

dioxide or liquid nitrogen to control temperature rise (Klettner, 1987). Chopping to lower temperatures targeted for a particular formulation may provide superior-quality products, as long as chopping time remained constant to prevent increased mechanical action that could produce unsatisfactory products.

CONCLUSION

FOR LOW-FAT FORMULATIONS, a chopping temperature of 12°C was the most effective. Generally formulation had the greatest effect on properties of frankfurters. For frankfurters made under the 40% rule, the formulation (AW level and reduced fat) was the most critical factor determining quality.

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Quality Characteristics of Restructured Beef Steaks Manufactured by Various Techniques

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ABSTRACT

Beef (9–11% fat) was restructured with each of six mechanical treatments including chunking, fiberizing, slicing, chunking + slicing, slicing + water, and tenderizing each in combination with addition of 0.5% sodium chloride (salt)/0.5% phosphate, 0.5% Na-alginate/0.5% Ca-lactate, 0.5% Na-pectate/0.5% Ca-lactate, or no additives (control). Beef steaks restructured with salt/phosphate had lower ($P<0.05$) purge losses, higher binding force and bind scores than control products. Steaks restructured with Na-alginate/Ca-lactate had lower cooking losses and higher bind scores than controls. Use of Na-pectate/Ca-lactate did not improve ($P>0.05$) purge loss, cooking loss, binding force or sensory properties. Chunked meat or mixtures of chunked and sliced meats in combination with salt/phosphate or Na-alginate/Ca-lactate resulted in steaks with acceptable bind and textural properties.

Key Words: restructured, beefsteak, lactate, alginate, pectate

INTRODUCTION

RESTRUCTURING TECHNOLOGY makes it possible to produce value-added meat products from low quality cuts. A wide variety of raw materials such as chuck muscles (Recio et al., 1987; Mann et al., 1990), beef riblifter (Ensor et al., 1990), shoulder clods (Liu et al., 1990), lamb (Field et al., 1984), mutton (Prasad et al., 1987), pork (Huffman et al., 1987; Trout et al., 1990), turkey (Ernst et al., 1989; Ensor et al., 1989) and chicken (Xiong and Brekke, 1991) have been used to produce restructured products. Traditionally, salt and phosphate have been used in restructured meat products because of beneficial effects on cohesion, cook yield, juiciness, and flavor (Clarke et al., 1987; Mann et al., 1989; Wheeler et al., 1990, Craig et al., 1991; Liu et al., 1992).

Consumer markets are demanding low-sodium meat products. Thus, partial replacement or substitution of sodium chloride with other binding agents may maintain or improve quality of restructured meat products while adapting to market needs. Some restructuring techniques do not use salt and phosphate to facilitate binding, relying on mechanical treatments such as thin slicing (Bradshaw et al., 1990; Cohen, 1990) with chunking (Huffman, 1981), kneading (Gagliardi, 1988) or ultrathin slicing (Fradin, 1991), and realigning muscle fibers (Guenther, 1989; Gibson, 1991). Some have used addition of binding agents such as Na-alginate/Ca-lactate (Means and Schmidt, 1986; Schmidt and Means, 1986), fibrinogen/thrombin (Paardekooper and Wijngaards, 1988), milk/egg/cracker (Polancic, 1990), and surimi-like materials prepared from beef by-products (Kenney et al., 1992). Sodium-pectate/Ca-lactate gels may be a potential binding system. Pectate, as well as alginate, can be made into gels in the presence of calcium ions at room temperature and has been used as a gelling agent in canned meat products (Hughes et al., 1980). Because of the variety of processing systems, restructured steaks may have widely variable sensory properties. Our objective was to evaluate several restructuring techniques including mechanical treatments such as chunking, tenderizing and slicing; and various restructuring ingredients such as salt/phosphate, Na-alginate/Ca-lactate and Na-pectate/Ca-lactate for production of restructured beef steaks.

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MATERIALS & METHODS

Sample preparation

Beef riblifter meat (NAMP #109B; Beef Rib, Blade Meat, 9–11% fat) was obtained from a local meat packing plant (Monfort Inc., Greeley, CO) and was used to evaluate six mechanical processes for restructuring. These included (1) chunking, (2) fiberizing, (3) tenderizing, (4) slicing, (5) chunking-slicing, and (6) slicing+water. Three binding agents included (1) 0.5% salt (NaCl)/0.5% phosphate (Heller's soluble phosphate WJ-0052, Heller Seasonings and Ingredients Inc., Bedford Park, IL), (2) 0.5% Na-alginate/0.5% encapsulated calcium lactate (Kelco, Chicago, IL) and (3) 0.5% Na-pectate (Sigma Chemical Co., St. Louis, MO)/0.5% encapsulated calcium lactate (Kelco, Chicago, IL) and a control (no additives). Thus, 24 treatments were studied. Usage levels (0.5%) of salt, phosphate, Na-alginate and Ca-lactate were based on previous research (Raharjo et al., 1994). Na-pectate was also incorporated at 0.5% to facilitate comparisons between treatments.

Chunked meat was prepared by grinding through a plate with 2.5 cm diameter openings (Hobart Mfg. Co., Troy, OH) without a cutting blade, providing relatively large chunks of meat with irregular shapes. Fiberized meat was obtained by manually slicing the meat with a knife into strands (1.5 cm × 1.5 cm and 10–20 cm long) parallel to the muscle fibers. Tenderized meat was obtained by passing 200–250 g portions through a blade tenderizer (Steakmaster model 401, Hobart-Federal Engineering Corp., Minneapolis, MN) twice (parallel and perpendicular to the muscle fiber). Sliced meat (8 mm thick) was prepared by slicing soft frozen meat with a meat slicer (Hobart Mfg. Co., Troy, OH). Chunked+sliced treatments consisted of 75% chunked and 25% sliced meat, while sliced+water treatments consisted of 90% sliced meat and 10% added water. Each batch (1250 g) of meat was mixed with binding agents for 2 min in a mixer (model K45SS, KitchenAid Inc., St. Joseph, MI) at a speed setting of 2. The mixture was placed in plastic bags (15 cm × 51 cm), sealed under vacuum (Multivac, Allgäu, Germany) and manually formed into logs (diameter 9 cm, length 35 cm). Meat logs were initially placed in a cooler at 4°C for 6 hr to provide adequate time for formation of alginate/calcium and pectate/calcium gels and subsequently stored in a freezer at −20°C for 48 hr prior to slicing into steaks (1.9 cm thick). Restructured beef steaks were individually vacuum-packaged and stored frozen at −20°C for ≈ 1 wk before further evaluation.

Purge loss

Three frozen restructured beef steaks from each treatment, individually vacuum-packaged, were thawed at 4°C. After 24 hr, steaks were removed from packages and surfaces were manually wiped with a paper towel to remove visible exudate. Purge was calculated as weight loss divided by initial weight, expressed as percentage.

Cooking loss

Three frozen restructured beef steaks from each treatment were tempered for 1 hr at 24–25°C prior to cooking on an Open Hearth Broiler™ (Farberware, Yonkers, NY) for 11 min on one side and another 10 min on the other side until internal temperature ≈ 71°C was reached. Cooking loss was calculated as weight loss due to cooking divided by initial weight of raw steak, expressed as percentage.

Table 1—Purge (%) loss of vacuum-packaged raw restructured beef steaks after thawing at 4°C for 24 hr[z]

Treatment	No additives (Control)	0.5% Salt (NaCl) 0.5% Phosphate	0.5% Na-Alginate 0.5% Ca-lactate	0.5% Na-Pectate 0.5% Ca-lactate
Chunked	5.3 ± 0.9^{Aa}	0.9 ± 0.7^{Ba}	4.3 ± 1.1^{Aa}	5.6 ± 0.6^{Ab}
Fiberized	4.0 ± 1.3^{Aa}	0.8 ± 0.2^{Ba}	3.5 ± 0.9^{ABa}	5.6 ± 1.2^{Ab}
Sliced	4.7 ± 0.3^{Aa}	0.9 ± 0.1^{Ba}	4.9 ± 0.7^{Aa}	5.6 ± 1.2^{Ab}
Sliced + chunked	6.3 ± 0.6^{Aa}	0.8 ± 0.3^{Ca}	3.7 ± 0.7^{Ba}	6.6 ± 0.8^{Ab}
Sliced + water	11.0 ± 1.7^{Ab}	1.9 ± 0.7^{Ca}	6.2 ± 1.0^{Ba}	9.7 ± 1.0^{Aa}
Tenderized	4.1 ± 0.4^{Aa}	0.9 ± 0.2^{Ba}	3.4 ± 0.7^{Aa}	4.5 ± 0.6^{Ab}

$^{A\ C}$ Means with different superscript within the same row are significantly ($P<0.05$) different.
$^{a\ b}$ Means with different superscript within the same column are significantly ($P<0.05$) different.
[z] Expressed as mean ± standard deviation.

Table 2—Cooking loss (%) of restructured beef steaks[z]

Treatment	No additives (Control)	0.5% Salt (NaCl) 0.5% Phosphate	0.5% Na-Alginate 0.5% Ca-lactate	0.5% Na-Pectate 0.5% Ca-lactate
Chunked	34.1 ± 1.9^{Ab}	30.2 ± 0.7^{ABab}	25.2 ± 2.6^{Ba}	34.4 ± 3.2^{Aa}
Fiberized	33.3 ± 0.5^{Ab}	33.5 ± 1.0^{Aa}	25.7 ± 4.9^{Ba}	32.3 ± 1.9^{ABa}
Sliced	34.9 ± 2.4^{Aab}	28.0 ± 2.8^{Bb}	29.1 ± 2.2^{ABa}	32.3 ± 1.2^{ABa}
Sliced + chunked	36.5 ± 1.3^{Aab}	31.1 ± 1.3^{Bab}	28.2 ± 0.9^{Ca}	33.7 ± 0.8^{ABa}
Sliced + water	39.5 ± 1.7^{Aa}	34.2 ± 2.5^{Aa}	33.3 ± 4.0^{Aa}	35.6 ± 1.5^{Aa}
Tenderized	33.5 ± 2.1^{Ab}	32.1 ± 1.6^{Aab}	26.1 ± 3.5^{Ba}	33.3 ± 0.7^{Aa}

$^{A\ C}$ Means with different superscript within the same row are significantly ($P<0.05$) different.
$^{a\ b}$ Means with different superscript within the same column are significantly ($P<0.05$) different.
[z] Expressed as mean ± standard deviation.

Binding force

Instrumental assessment was made on the degree to which particles of restructured steaks physically adhere to each other within the cooked product. We used a test cell and a 2 cm diameter steel ball probe (modification of Field et al., 1984) on a J.J. Lloyd tensile testing machine (Model T5002, Pacific Scientific, Santa Ana, CA), at 24–25°C. Settings were: load cell 100 N, sensitivity 1.0, and crosshead speed 110 mm/min. Three steaks (those used for cooking loss evaluation) from each treatment were tested. The peak height was measured (mm) and converted to Newtons.

Sensory evaluation

Five trained panelists evaluated the restructured steaks. Panelists were trained following the procedure of Means and Schmidt (1986). Steaks were cooked as described and served to panelists immediately after cooking. In every session, only six treatments, randomly selected, were evaluated. Each panelist was supplied with a plate, a steak knife, a fork, a glass of cold tap water, and a disposal cup. Panelists received ≈ 30g of steak from each treatment. They were asked to rinse their mouths with cold tap water before evaluating each sample. Restructured beef steaks were cut with a knife and received a score of 1 for very weak bind between meat chunks (comparable to a ground beef patty), to 6 for very strong bind (comparable with intact muscle steak). Juiciness (score 1: very dry to 6: very juicy), flavor (score 1: very undesirable to 6: very desirable), texture (score 1: ground beef-like texture to 6: steak-like texture) were also evaluated. Because this was a trained panel, panelists were instructed to base flavor desirability scores on presence of any off-flavors associated with the restructured steak, instead of personal preference. Uniformity of interior color of the cooked steak was also evaluated. Particle to particle variation (color) can exist in restructured meat products. A score of 1 indicated very little cooked color uniformity, a score of 6 indicated very good cooked color uniformity.

Statistical analysis

The experiment was replicated twice with meat from a separate box and triplicate measurements were conducted in each replicate. Purge, cook loss, binding force and sensory evaluation data were presented as means along with standard deviations. Significant differences among treatments within each type measurement were determined by analysis of variance. Treatment means were separated by Tukey's test (Steel and Torrie, 1980) when a significant ($P<0.05$) F-test for main effects was found.

RESULTS & DISCUSSION

Purge loss

Beef steaks restructured with salt/phosphate had lower ($P<0.05$) purge than steaks restructured with no additives (control) (Table 1). Addition of NaCl typically weakens interaction between oppositely charged groups of myofibrillar proteins, resulting in increased swelling and water holding capacity (Hamm, 1986). The addition of Na-pectate/Ca-lactate had no ($P>0.05$) effect on purge as compared with controls. Purge in steaks restructured with Na-alginate/Ca-lactate was not ($P>0.05$) different from controls, except for sliced+chunked and sliced+water treatments which had less ($P<0.05$) purge. Mechanical treatment had no ($P>0.05$) effect on purge in steaks restructured with salt/phosphate or Na-alginate/Ca-lactate. Treatments containing 10% added water had higher ($P<0.05$) purge in steaks restructured with Na-pectate/Ca-lactate or no additives.

Cooking loss

Addition of Na-alginate/Ca-lactate reduced ($P<0.05$) cook loss, except for sliced and sliced+water treatments which were not different from corresponding controls (Table 2). Treatments formulated with salt/phosphate and sliced or sliced+chunked meat had lower ($P<0.05$) cooking loss than controls. Therefore, steaks restructured with salt/phosphate had higher ($P<0.05$) cook yields when they were prepared from sliced or sliced +chunked meat. Previous studies have indicated that use of salt and phosphate increased cook yield in restructured steaks (Mann et al., 1989; Wheeler et al., 1990, Craig et al., 1991). Cook loss or cook yield was related to cooking method (Quenzer and Donnelly, 1982), oven temperature, relative humidity, sample dimensions (Bengtsson et al., 1976) and internal temperature (Tanchotikul et al., 1989). Steaks prepared with no additives and added water had higher ($P<0.05$) cook losses than those prepared with chunked, fiberized or tenderized meats. Various mechanical treatments did not affect ($P>0.05$) cook loss when steaks were restructured using Na-alginate/Ca-lactate or Na-pectate/Ca-lactate.

Binding force

Beef steaks restructured with salt/phosphate had higher ($P<0.05$) binding force than controls (Table 3). This was in agreement with previous studies which reported that addition of salt (0.5 to 0.75%) and phosphate (up to 0.5%) were required to achieve satisfactory bind in restructured beef steaks (Coon et al., 1983). Salt and phosphate induce solubilization of myofibrillar proteins during mixing and this exudate serves as binding agent upon cooking (Ghavimi et al., 1987). Addition of Na-pectate/Ca-lactate did not improve ($P>0.05$) binding force com-

Table 3—Binding force[y] (Newtons) of cooked restructured beef steaks[z]

Treatment	No additives (Control)	0.5% Salt (NaCl) 0.5% Phosphate	0.5% Na-Alginate 0.5% Ca-lactate	0.5% Na-Pectate 0.5% Ca-lactate
Chunked	19.9 ± 1.2^{Ba}	48.0 ± 9.4^{Aa}	36.7 ± 1.3^{ABab}	27.9 ± 13.3^{ABa}
Fiberized	31.5 ± 12.2^{Ba}	54.9 ± 5.8^{Aa}	31.2 ± 10.1^{Bab}	28.3 ± 3.8^{Ba}
Sliced	19.5 ± 2.4^{Ba}	55.7 ± 14.2^{Aa}	24.9 ± 2.6^{Bb}	15.2 ± 3.8^{Ba}
Sliced + chunked	29.6 ± 9.1^{Ba}	56.1 ± 14.4^{Aa}	44.0 ± 4.2^{ABa}	23.1 ± 7.7^{Ba}
Sliced + water	15.1 ± 0.9^{Ca}	47.6 ± 5.9^{Aa}	26.0 ± 2.4^{Bb}	18.4 ± 2.0^{BCa}
Tenderized	20.8 ± 2.9^{Ba}	46.8 ± 10.5^{Aa}	28.7 ± 5.8^{Bb}	14.8 ± 2.2^{Ba}

[A C] Means with different superscript within the same row are significantly ($P<0.05$) different.

[a b] Mean with different superscript within the same column are significantly ($P<0.05$) different.

[y] Force (Newtons) to penetrate cooked steak with a 2 cm diameter steel ball.

[z] Expressed as mean ± standard deviation.

Table 4—Sensory evaluation[y] of cooked restructured beef steaks[z]

Treatment	No additives (Control)	0.5% Salt (NaCl) 0.5% Phosphate	0.5% Na-Alginate 0.5% Ca-lactate	0.5% Na-Pectate 0.5% Ca-lactate
		Juiciness		
Chunked	3.6 ± 1.5^{ABa}	5.0 ± 0.0^{Aa}	5.0 ± 0.0^{Aa}	2.8 ± 0.4^{Ba}
Fiberized	3.2 ± 1.3^{Aa}	4.2 ± 1.1^{Aa}	4.8 ± 0.8^{Aa}	3.8 ± 1.1^{Aa}
Sliced	3.0 ± 0.7^{Aa}	3.6 ± 0.9^{Aa}	4.6 ± 0.9^{Aa}	3.8 ± 1.1^{Aa}
Sliced + chunked	3.8 ± 0.8^{Aa}	4.8 ± 0.8^{Aa}	5.0 ± 1.2^{Aa}	3.4 ± 1.1^{Aa}
Sliced + water	3.4 ± 1.1^{Ba}	5.0 ± 0.0^{Aa}	5.4 ± 0.5^{Aa}	3.2 ± 1.1^{Ba}
Tenderized	3.4 ± 1.1^{Aa}	4.8 ± 0.8^{Aa}	4.4 ± 0.5^{Aa}	4.4 ± 1.1^{Aa}
		Bind		
Chunked	1.4 ± 0.5^{Ba}	5.0 ± 0.7^{Aa}	5.6 ± 0.5^{Aa}	1.6 ± 0.9^{Ba}
Fiberized	1.6 ± 1.3^{Ba}	4.8 ± 0.4^{Aa}	4.2 ± 1.1^{Aab}	1.6 ± 0.5^{Ba}
Sliced	1.4 ± 0.9^{BCa}	5.0 ± 0.7^{Aa}	3.2 ± 1.6^{ABb}	1.2 ± 0.4^{Ca}
Sliced + chunked	2.0 ± 1.0^{Ba}	5.4 ± 0.5^{Aa}	4.0 ± 1.6^{Aab}	2.0 ± 0.7^{Ba}
Sliced + water	2.4 ± 0.5^{BCa}	5.0 ± 0.0^{Aa}	3.6 ± 1.1^{Bab}	1.6 ± 0.5^{Ca}
Tenderized	1.2 ± 0.4^{Ba}	5.0 ± 0.7^{Aa}	4.2 ± 0.8^{Aab}	1.4 ± 0.5^{Ba}
		Flavor		
Chunked	3.8 ± 1.3^{ABa}	4.8 ± 1.1^{Aa}	4.6 ± 1.1^{Aa}	2.0 ± 1.4^{Ba}
Fiberized	3.4 ± 1.1^{Aa}	4.8 ± 0.8^{Aa}	3.4 ± 1.7^{Aa}	3.2 ± 0.4^{Aa}
Sliced	3.6 ± 1.1^{Aa}	4.4 ± 0.5^{Aa}	4.4 ± 0.5^{Aa}	3.2 ± 1.1^{Aa}
Sliced + chunked	4.0 ± 1.0^{Aa}	4.2 ± 0.8^{Aa}	3.2 ± 1.5^{ABa}	1.8 ± 1.3^{Ba}
Sliced + water	4.2 ± 0.8^{Aa}	4.2 ± 0.8^{Aa}	3.6 ± 0.5^{Aa}	2.8 ± 0.8^{Aa}
Tenderized	3.8 ± 0.4^{ABa}	5.4 ± 0.9^{Aa}	3.2 ± 1.8^{Ba}	2.8 ± 1.3^{Ba}
		Texture		
Chunked	3.2 ± 0.8^{BCab}	4.6 ± 0.9^{ABa}	5.0 ± 1.2^{Aa}	2.4 ± 0.5^{Ca}
Fiberized	4.2 ± 0.8^{Aa}	4.8 ± 0.4^{Aa}	4.4 ± 1.3^{Aa}	3.6 ± 0.9^{Aa}
Sliced	3.2 ± 0.8^{ABab}	4.4 ± 1.3^{Aa}	3.2 ± 1.3^{ABa}	2.4 ± 0.5^{Ba}
Sliced + chunked	3.6 ± 1.1^{Aab}	4.4 ± 0.9^{Aa}	3.6 ± 1.8^{Aa}	2.2 ± 0.8^{Aa}
Sliced + water	3.2 ± 1.6^{Aab}	4.6 ± 1.1^{Aa}	4.0 ± 0.7^{Aa}	3.2 ± 1.6^{Aa}
Tenderized	1.8 ± 0.8^{Ab}	3.6 ± 1.9^{Aa}	2.8 ± 0.4^{Aa}	2.0 ± 0.0^{Aa}

[A C] Means with different superscript within the same row are significantly ($P<0.05$) different.

[a b] Means with different superscript within the same column are significantly ($P<0.05$) different.

[y] Juiciness (1: very dry and 6: very juicy), bind (1: very weak bind and 6: very strong bind). Flavor (1: very undesirable and 6: very desirable) and texture (1: ground beef-like texture and 6: whole muscle steak-like texture).

[z] Expressed as mean ± standard deviation.

pared to controls. Although Na-pectate could form a gel with calcium (Hughes et al., 1980), a cohesive gel did not form under our conditions. The mechanical strength of calcium pectate gel is, in part, determined by the degree of esterification of pectin and the pH of the mixture (Gemeiner et al., 1991). Further study is needed to determine proper types of pectate and calcium sources and appropriate conditions for pectate/calcium gel formation. Use of Na-alginate/Ca-lactate increased ($P<0.05$) binding force in steaks prepared from sliced meat with added water only, as compared with corresponding controls. As a result of varying shapes and sizes of meat particles, binding force in restructured steaks could show relatively large variations (Berry and Civille, 1986). Increasing the level of Na-alginate (from 0.5% to 1.0%) might increase the binding force as reported by Clarke et al. (1988) and Ensor et al. (1989). The maximum level of Na-alginate and Ca-lactate allowed in restructured meat products is 1.5% (USDA FSIS, 1986). The various mechanical treatments had no ($P>0.05$) effect on binding force, except for steaks restructured with Na-alginate/Ca-lactate and a mixture of sliced and chunked meat. These had significantly higher binding force than that of steaks prepared with sliced, sliced+water and tenderized meat. Preparation of restructured steaks by combining meat cuts with different shapes had been reported (Huffman 1981 and Cohen 1990).

Sensory evaluation

Steaks restructured with Na-pectate/Ca-lactate had similar ($P>0.05$) juiciness as those restructured with no additives (control) (Table 4). Those restructured with salt/phosphate or Na-alginate/Ca-lactate also had similar ($P>0.05$) juiciness, except for steaks prepared with added water, which were juicier than controls. This agreed with Ensor et al. (1989) who reported that addition of Na-alginate/Ca-lactate binder with no added water did not change ($P>0.05$) juiciness of restructured turkey meat as determined by panelists. All mechanical treatments had no ($P>0.05$) effect on cooked product juiciness. However, addition of water (10%) along with salt/phosphate or Na-alginate/Ca-lactate may be useful to increase restructured steak juiciness.

Use of Na-alginate/Ca-lactate improved ($P<0.05$) bind scores of restructured beef steaks as compared with controls. Panelists were more effective in assessing this than was the instrumental method. Steaks restructured with salt/phosphate had higher ($P<0.05$) bind scores than controls. This was in agreement with instrumental binding force results. Steaks restructured with Na-pectate/Ca-lactate were not different from the control for bind.

Mechanical treatment had no ($P>0.05$) effect on flavor of steaks restructured with no additives, salt/phosphate, Na-alginate/Ca-lactate or with Na-pectate/Ca-lactate. Thus, addition of

water (10%) did not affect the flavor of restructured beef steaks. Addition of Na-pectate/Ca-lactate tended to decrease flavor acceptability of restructured steaks. The source of objectionable flavor may be the Na-pectate, or a product of a reaction between Na-pectate and meat components. The use of Na-alginate/Ca-lactate as restructuring components did not ($P>0.05$) affect flavor acceptability as compared to controls. Previous studies have indicated that the flavor of restructured beef (Means et al., 1987) or turkey meat (Ensor et al., 1989) prepared with Na-alginate/Ca-lactate binder (0.5 to 1.0%) did not differ ($P>0.05$) from controls. Similarly, panelists found no differences in flavor desirability between steaks formulated with salt/phosphate and controls.

Texture of restructured steaks could range from "ground beef-like" to "steak-like." The texture of steaks restructured with salt/phosphate or Na-pectate/Ca-lactate was not different ($P>0.05$) from the control. Addition of Na-alginate/Ca-lactate also had no ($P>0.05$) effect on texture of the restructured steak, except for the chunked treatment which was evaluated as superior to the control. The various mechanical treatments had no effect ($P>0.05$) on texture of steaks formulated with salt/phosphate, Na-alginate/Ca-lactate or Na-pectate/Ca-lactate. In control treatments where no additives were used, tenderized meat resulted in steaks with ground beef-like texture. Use of fiberized meat seemed to result in desirable steak-like texture. Berry et al. (1987) also recommended larger sized meat pieces to improve product texture. Sliced meat may also be used in combination with chunked meat to improve product tenderness while maintaining acceptable texture. Various mechanical treatments and restructuring components did not significantly affect color uniformity (data not shown) of cooked restructured beef steaks. However, on a 6-point scale (1: very poor cooked color uniformity and 6: very good cooked color uniformity), color uniformity scores ranged from 3.2 to 4.2, 2.4 to 3.6, 3.6 to 5.0, and 2.6 to 4.0 for control, salt/phosphate, Na-alginate/Ca-lactate, and Na-pectate/Ca-lactate treatments, respectively.

Techniques to manufacture restructured meat products vary with respect to mechanical treatments and binding ingredients. Our results indicated that chunked meat or mixtures of chunked and sliced meats in combination with salt/phosphate or Na-alginate/Ca-lactate resulted in steaks with acceptable physical and sensory properties. Utilization of Na-pectate/Ca-lactate as binding ingredients did not improve quality of restructured beef.

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Improved Calpain Assay Using Fluorescein Isothiocyanate-Labeled Casein

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ABSTRACT

A sensitive assay has been developed using a fluorescein isothiocyanate (FITC)-labeled casein substrate. Fluorescence of TCA-soluble FITC-labeled peptides was measured with an excitation wavelength of 490 nm and an emission wavelength of 513 nm. Fluorometric results in a time course assay correlated highly (r=0.985) with standard calpain assay (absorbance of TCA-soluble peptides at 278 nm) results. Fluorescence assay results correlated highly with calpain activity (r=0.997) in an activity titration assay and were substantially more sensitive than the standard method. Using FITC-labeled casein provides a simple, sensitive assay for μ- and m-calpain.

Key Words: calpain, assay, fluorescein isothiocyanate, casein

INTRODUCTION

THERE IS MUCH EVIDENCE that the calpain enzyme system is involved in postmortem proteolysis of meat proteins and meat tenderization (Koohmaraie, 1994). The calpains can also be activated early postmortem to have a beneficial effect on meat tenderness. Two forms of calpain are present in beef muscle. One form requires millimolar concentrations of calcium (m-calpain) and another requires only micromolar concentrations of calcium (μ-calpain) for activation (Koohmaraie, 1992). Shackelford et al. (1991) have shown that calpastatin, the endogenous calpain inhibitor, could be a useful predictor of meat tenderness. Thus, the calpain enzyme system may have a function in postmortem proteolysis and tenderization.

A limiting factor in understanding the regulation of the calpain enzyme system is the size of tissue samples required for a calpain activity assay. Existing assay techniques require 0.2 to 0.4 units of caseinolytic activity (Koohmaraie, 1990). Such assay procedures are widely accepted, but require a minimum of 10g muscle tissue. The calpain preparation technique would be more advantageous if smaller tissue samples and cell extracts could be used to define the regulation of calpain. However, such calpain preparation techniques may not be adequately evaluated unless a more sensitive calpain activity assay is developed.

Fluorescein isothiocyanate (FITC) is a fluorescent label for casein substrates used in micro-assays for trypsin, chymotrypsin, elastase and subtilisin (Twining, 1984). FITC is an effective label for casein because it is highly fluorescent and reacts with amino groups of casein to form a fluorescein thiocarbamoyl (FTC) derivative. The fluorescent nature of FITC makes it possible to detect casein peptides resulting from proteolytic degradation using spectrofluorometric methods. The use of FITC-labeled casein in a calpain assay has been reported (Wolfe et al., 1989). Our objective was to alter fluorescence measurement techniques, and to demonstrate an increased sensitivity.

MATERIALS & METHODS

Substrate preparation

One gram of casein was added per dL of 50 m*M* sodium carbonate containing 150 m*M* sodium chloride, pH 9.5; FITC was added at 20, 40, or 60 mg/dL of casein buffer and stirred at room temperature (≈23°C) for 2 hr for the formation of FTC-casein. The effectiveness of several dialysis schedules in the removal of free FITC was evaluated, and the most efficient removal of unbound FITC was applied. Free FITC was removed by dialyzing twice (24 h) against suspensions of charcoal (5 g/L). The FTC-casein was dialyzed once against 50 m*M* Tris, pH 8.5, followed by two 24-hr dialysis periods against 100 m*M* Tris, pH 7.5, to yield a buffered substrate. All dialyses were in the dark at 4°C.

Calpain assay substrate was prepared by diluting prepared FTC-casein buffer with an equal volume of 10 m*M* $CaCl_2$, 100m*M* Tris, pH 7.5. All assays included controls where 20 m*M* EDTA was substituted for the $CaCl_2$. The resulting assay substrate contained 5 m*M* $CaCl_2$, 100 m*M* Tris, pH of 7.5. 2-mecaptoethanol (0.05%) was added immediately before the assay. Protein and FITC content of the substrate was determined by the Lowry method (Lowry et al., 1951), and by measuring absorbance of the substrate at 490 nm using the molecular extinction coefficient 61,000 $M^{-1}\cdot cm^{-1}$ (Brodrick et al., 1980).

Assay procedure

Ion-exchange chromatography was used to prepare partially purified m-calpain (Koohmaraie, 1990). Pooled calpain fractions were incubated with 0.9 mL assay substrate at 25°C. The reaction was stopped following the incubation period with addition of 1.2 mL 5% trichloroacetic acid (TCA). The TCA-insoluble protein was removed by centrifugation at 2000 × G for 30 min. The supernatant was further clarified with a second, identical centrifugation.

A 500-μL sample of supernatant was diluted to 3.0 mL with 500 m*M* Na_2HPO_4, pH 8.5, buffer. Fluorescence was measured using an excitation wavelength of 490 nm and an emission wavelength of 513 nm. These wavelengths were selected by identifying the maximum fluorescent yield of FITC standard (20 ng/mL) at pH 8.5 (Fig. 1). FTC-casein displayed the same emission scan. A FITC standard containing 10 ng FITC/mL was used to calibrate the spectrofluorometer prior to each assay. Fluorescence of blanks [(EDTA + calpain) − (EDTA + elution buffer)] was subtracted from the fluorescence of the assay supernatant [($CaCl_2$ + calpain) − ($CaCl_2$ + elution buffer)] for fluorescent measurement of proteolytic activity.

Assay comparison

To evaluate the reliability of the assay, caseinolytic activity of beef muscle m-calpain was determined by measuring absorbance of TCA-soluble peptides (Koohmaraie, 1990) in solution, and was compared to fluorescence measurements in a 110-min time course study. Thus, 0.3 mL of pooled beef muscle m-calpain was added to 0.9 mL of assay substrate and incubated from 10–110 min.

In a separate experiment, activity of m-calpain samples was determined by measuring absorbance of TCA-soluble peptides at 278 nm. One unit of activity was defined as the amount of calpain that catalyzed an increase of 1.0 absorbance unit at 278 nm in 60 min at 25°C. Partially purified calpain was diluted to contain increasing levels of activity from 0.018 to 0.20 units of activity/300 μl. aliquot. This experiment used 60 min incubation for all samples.

A final experiment was conducted to investigate the sensitivity of the fluorescent assay. Pooled m-calpain from DEAE-Sephacel was divided in aliquots to provide a range of activity from 0.02 to 0.04 units/mL. Each of these aliquots (1 mL) was incubated with 1 mL FTC-casein substrate containing 5 mg/mL casein and 27 μg FITC/mg casein. Fluorescence and absorbance measurements were as described, except that 750 μL were diluted with 2.25 mL 500 m*M* Na_2HPO_4, pH 8.5.

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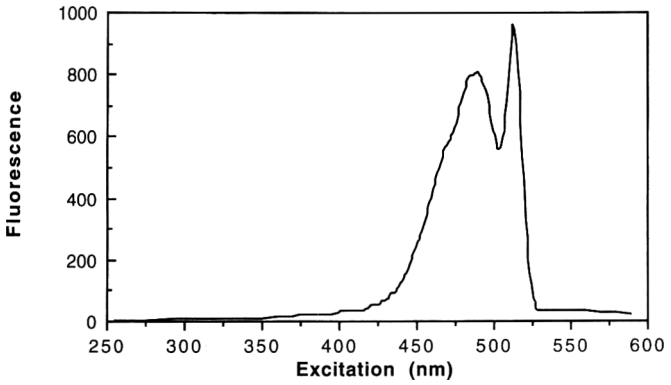


Fig. 1—Excitation scan of FITC in 500 mM Tris, pH 8.5 solution. Emission = 513 nm. Maximum fluorescent yield detected at 490 nm. The peak at 513 nm occurs when excitation λ = emission λ.

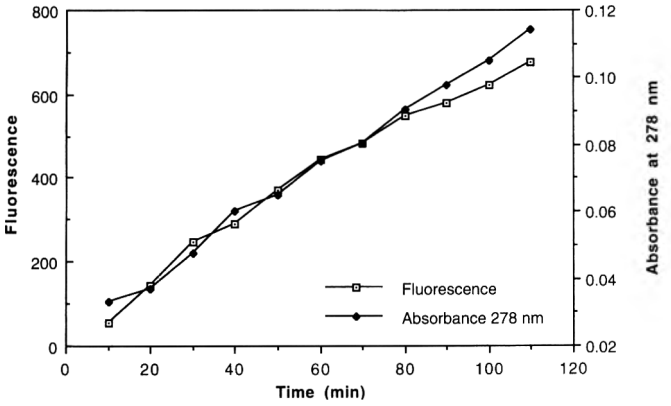


Fig. 2—Fluorescence and absorbance values from a time course assay correlated highly (r=0.985). Fluorescence Standard error of the mean (SEM)=1.8. Absorbance SEM=0.001.

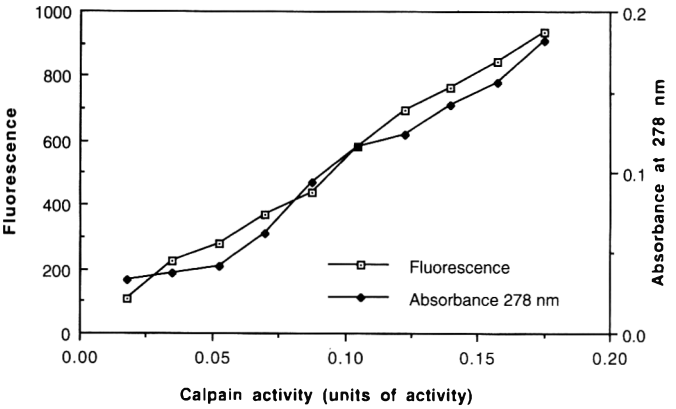


Fig. 3—Fluorescence and absorbance results from assay of fractions containing increasing proteolytic activity. Fluorescence r=0.997, SEM=1.8. Absorbance r=0.987, SEM=0.003.

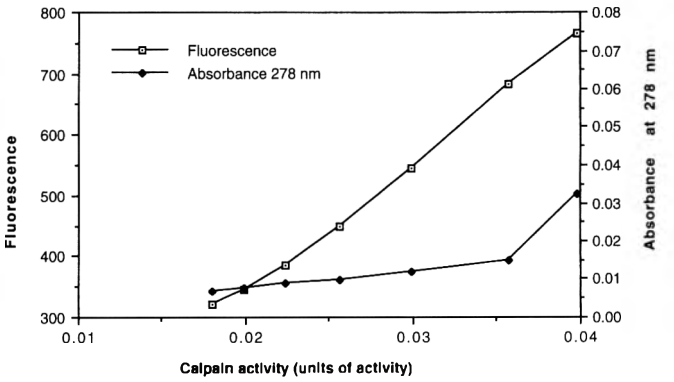


Fig. 4—Fluorescence and absorbance results from an assay of m-calpain aliquots at very low levels of activity. Fluorescence r=0.999, SEM=1.8. Absorbance r=0.85, SEM=0.004.

Statistical analysis

Data were analyzed by analysis of variance and linear regression with the GLM procedure of SAS Institute, Inc. (1989). All assays included triplicate samples and were repeated three times.

RESULTS & DISCUSSION

SUBSTRATES PREPARED BY LABELING with increasing concentrations of FITC (20-60 mg/dL buffer) indicated that increasing concentrations of FITC increased the sensitivity. Preliminary results revealed that addition of 60 mg of FITC/dL buffer diminished precision of fluorescence results. This may have been due to incomplete removal of FITC during dialysis. Therefore, 40 mg FITC/dL buffer was used for further development. This produced a FTC-casein buffer containing ≈25 µg FITC/mg casein. Frozen storage of the substrate increased stability of the assay medium as compared to refrigerated storage. This may have been due to FITC dissociation from amino groups of casein during refrigerated storage. Failure to remove unbound FITC from the assay buffer during substrate preparations led to high blank fluorescence readings and inconsistent results. The TCA supernatant had to be thoroughly clarified to avoid erroneous fluorescence results. If the TCA supernatant was not completely neutralized, low fluorescence was obtained, which would result in underestimating calpain activity.

Fluorescence of neutralized TCA-soluble peptides was measured with an excitation of 490 nm and an emission of 513 nm as described since the excitation scan (Fig. 1) defined those wavelengths as optimum for sensitive detection. Others using this type assay measured fluorescence with an excitation of 287 nm and an emission of 518 nm. The excitation scan of a 20 ng/mL FITC, pH 8.5, showed no fluorescence using those conditions. The increased sensitivity with excitation of 490 nm and emission of 513 nm enabled the identification and quantification of proteolytic activity in chromatographic fractions and pooled samples when calpain activity was limited. In the time course experiment, absorbance and fluorescence values correlated highly (r = 0.985) indicating the fluorescence assay was reliable (Fig. 2). Data also indicated that the amount of substrate for the assay was not limiting.

The second experiment revealed that both absorbance (r = 0.987) and fluorescence correlated highly (r = 0.997) with the known activity of calpain in the assay (Fig. 3). Absorbance values of TCA-soluble peptides were not different at the lowest 3 levels of activity ($P > 0.1$). This indicated that increased proteolytic activity was not detected until 0.1 units of m-calpain activity were available. Fluorescence values indicated increasing activity even at the 0.02 units. This indicated that the fluorometric assay was at least five times more sensitive than the absorbance assay and better suited to determine calpain activity in isolates from small tissue samples. Results of the final experiment (Fig. 4) showed a clear advantage in sensitivity over the standard assay. Calpain activity was detected at the lowest level (0.018 units) assayed. Conversely, the absorbance of TCA-soluble peptides did not detect an increase in calpain activity through the range measured.

Our fluorescence assay makes it possible to evaluate calpain purification from small tissue samples or cell extracts. Use of the fluorescein label brings this assay into the sensitivity range of radioactive assays (Twining, 1984). The fluorescent assay, however, does not create waste disposal or health hazard problems.

CONCLUSIONS

A SIMPLE CALPAIN ASSAY that was at least 5× more sensitive than existing procedures was developed. This calpain assay will

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Tetrathiafulvalene-mediated Biosensor for L-lactate in Dairy Products

ASHOK MULCHANDANI, AMARJEET S. BASSI, and ANDREW NGUYEN

ABSTRACT

A tetrathiafulvalene (TTF)-mediated, amperometric biosensor was developed for the specific determination of L-lactate. L-lactate oxidase was immobilized by adsorption and retained on the surface of a carbon-paste electrode using an electro-polymerized film of 1,3-phenylenediamine-resorcinol. The response of the biosensor was maximum at pH 7.0 and 30°C. Response was linear up to 0.6 mM lactate and had a minimum detection limit of 56 µM. Response time was less than 90 sec. When stored at 4°C response was stable for > 36 days and subsequently decreased. The sensor was unresponsive after 96 days. This enzyme-based biosensor was suitable for determining lactate concentrations in milk and yogurt.

Key Words: tetrathiafulvalene, lactate oxidase, biosensor, L-lactate, dairy products

INTRODUCTION

LACTIC ACID CONCENTRATION in milk, yogurt and other milk products is an important index of quality (Lawrence, 1975). In the food industry, D- and/or L-lactic acid monitoring can be used as an indicator of food spoilage (Turner, 1987; Korkeala et al., 1990; Shu et al., 1993). Determination of lactic acid is also important in medicine (Smith, 1991), sports medicine (Kulys et al., 1993), and biotechnology.

Traditionally, techniques such as enzymatic assays, chemical methods (Barkar and Summerson, 1941; Burini, 1943), gas chromatography (Carlsson, 1973), and high-pressure liquid chromatography (HPLC) have been used to measure lactic acid. Because of obvious advantages of rapid analysis and simplicity and applications of on-line process monitoring, there has been great interest in development of novel biosensor devices for lactate.

Biosensors based on both whole cells and enzymes have been reported. In the whole cell-based biosensor for lactate, *Acetobacter pasteurianus* microorganism was combined with a dissolved-oxygen electrode (Luong et al., 1989). Like the other whole cell-based biosensors it was not specific and did not differentiate between D- and L+ isomers. Additionally, since it was based on measurement of oxygen consumption, the performance of the biosensor would be affected by such parameters as pH, ionic concentration, temperature and partial pressure of oxygen, which would affect oxygen solubility.

Several L-lactate enzyme electrodes based on lactate dehydrogenase (LDH) (Blaedel and Engstrom, 1980), cytochrome b_2 (Scheller et al., 1987), lactate monoxygenase (LMO) (Mascini et al., 1984), and lactate oxidase (LOD) have been reported. Enzyme electrodes based on LDH and cytochrome b_2 however have short-term stability, because of fouling of electrodes due to oxidation products of NADH or mediators (Scheller et al., 1987) and high cost due to the need to supply NAD, a costly and unstable co-substrate, in the assay buffer (Kulys et al., 1993). Both the LMO- and LOD-based biosensors have a disadvantage due to dissolved oxygen fluctuations resulting from variations in pH, temperature, ionic strength and partial pressure. Additionally, LOD enzyme electrodes based on hydrogen peroxide measurement may have interference due to non-specific electro-oxidation of compounds such as ascorbic acid, uric acid, glutathione, etc., at the relatively high potential (0.6–0.7V vs Ag/AgCl reference) required for electrochemical oxidation of hydrogen peroxide.

The problems of oxygen fluctuations and required high potential could be overcome by using chemically modified enzyme electrodes. In such an electrode the electron acceptor dioxygen would be replaced by a low-molecular-weight species, such as ferrocene, tetrathiafulvalene (TTF), tetracyanoquinodimethane (TCNQ), etc. These could shuttle electrons between the redox center of the LOD and the working electrode and allow operation of the enzyme electrodes at much lower potentials where effects of interferences would be minimized.

TTF is low-molecular-weight and shuttles electrons between the redox center of the enzyme and the working electrode thereby eliminating a need for oxygen as electron acceptor. TTF has extremely low solubility in aqueous solutions and low potential would be required for regenerating the mediator. The low solubility would enable its retention in the carbon paste matrix and other surfaces by adsorption. TTF-mediated enzyme electrodes have been reported for detection of glucose (Gunasingham and Tan, 1990), glutamate (Almeida and Mulchandani, 1993) and other compounds. Previously, an amperometric TTF-mediated lactate electrode using lactate oxidase adsorbed on carbon foil was reported for use as a disposable lactate enzyme sensor (Palleschi and Turner, 1990).

Our objective was to develop a re-usable amperometric biosensor based on TTF as a mediator in a carbon paste electrode and determine its application for monitoring L-lactate in spoiled milk and yogurt.

The reaction scheme for the TTF-mediated biosensor can be represented as follows:

$$\text{L-lactate} + \text{LOD (oxidized)} \rightarrow \text{pyruvate} + \text{LOD (reduced)} \quad (1)$$

$$\text{LOD (reduced)} + 2\text{TTF}^{+} \rightarrow \text{LOD (oxidized)} + 2\ \text{TTF} \quad (2)$$

$$2\text{TTF (at electrode)} \rightarrow 2\text{TTF}^{+} + 2e^{-} \quad (3)$$

The current produced in the reoxidation of TTF is correlated to the lactate concentration.

MATERIALS & METHODS

Chemicals

L-lactate oxidase (E.C. 1.1.3.2) from *Pediococcus* species, L-lactic acid, horseradish peroxidase, and 4-aminoantipyrine were purchased from Sigma Chemical Company, St. Louis, MO. Graphite powder (Grade #38), TTF, mineral oil (white, light), resorcinol and 1,3-phenylenediamine were purchased from Aldrich (Milwaukee, WI). Phenol was obtained from Fisher Scientific (Pittsburgh, PA). All other chemicals and buffers were analytical grade or better. Milk and yogurt were obtained from a local supermarket.

Preparation of TTF-carbon paste electrode

The carbon paste electrode was prepared as follows: To 25 mg graphite powder, 1 µmole mg^{-1} graphite of TTF (previously dissolved in

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acetone) was added and the mixture uniformly blended with a stainless steel spatula. After residual traces of acetone had evaporated, 20% (w/w) of mineral oil was added and the resulting paste mixed to an even consistency. The paste was then packed into the top half of a well of a Kel-F electrode holder (Bioanalytical Systems Inc. (BAS), W. Lafayette, IN, Part MF 2010, inside diameter 3 mm) in which the lower half had been previously packed with a similar carbon paste comprised of graphite powder plus mineral oil (20% w/w) but without the TTF. The surface of the packed electrode was then polished to a smooth shiny finish by gently rubbing over a weighing paper. A 0.5 mm deep dent was made (using a specially machined plexiglass tool) in this polished surface to prevent spreading of enzyme solution onto the electrode body during subsequent enzyme adsorption.

Enzyme immobilization

In order to increase the loading of the enzyme by adsorption onto the relatively hydrophobic carbon paste electrode surface, the electrode tip was immersed into a gently stirred solution of Triton-X surfactant (0.1% v/v in distilled water) for 30 min. After wiping excess surfactant carefully from all areas surrounding the well, a small volume (2.5 to 10 µL) of lactate oxidase (of appropriate activity) dissolved in 0.1M phosphate buffer (pH 7.0) was carefully dropped on the working surface of the electrode and allowed to adsorb for 1.5 hr. Subsequently, the carbon paste electrode was made the working electrode in an electrochemical cell containing a deaerated solution of 1.5 mM each of resorcinol and 1,3-phenylenediamine. The working electrode potential was cycled between 0 and +0.6 V at a scan rate of 20 mV/sec for a total of 8 cycles to deposit a polymer film behind which the adsorbed enzyme was entrapped. The formation of a polymer film could be confirmed visually as a white colored film. The choice of monomers and conditions of polymerization were based on results previously reported. The electropolymerized film thus obtained provided good protection and stability to the enzyme and also prevented electrode fouling (Almeida and Mulchandani, 1993). Cyclic voltammograms were recorded on the X-Y recorder and during the electropolymerization an inert (argon) atmosphere was maintained in the electrochemical cell. Finally, the electrode was washed by immersing the tip into phosphate buffer (0.1M, pH 7.0) and by gently stirring for 30 min to remove any unbound materials and monomers.

Optimization of operating parameters

All optimization studies were conducted in a 30-mL jacketed electrochemical cell connected to a constant-temperature water bath. The response (output current increase) of the electrode was optimized with respect to pH (between 6.5 and 8.0 in 0.1M phosphate buffer), temperature (between 20 and 37°C) and number of units of lactate oxidase (between 2.15 and 12.75 U) immobilized on the electrode surface. All experiments were done in duplicate.

Electrochemical methods

Cyclic voltammetry and amperometric (i.e., constant potential) measurements were performed using a Bioanalytical Systems (BAS) voltammograph (Model CV 27) coupled to a low current module (BAS, Module PA1), and an X-Y-t chart recorder (BAS, MF 8050). All experiments were conducted using a 3-electrode electrochemical cell inside a Faraday cage (BAS, Model C2 cell stand), with a Ag/AgCl reference electrode (BAS, MF 2063), and a platinum wire auxiliary electrode (BAS, MF 1032). All solutions were deaerated for 30 min prior to use by argon sparging and used under an argon blanket. In all amperometric studies contents of the electrochemical cells were mixed by magnetic stirring bar.

Determination of L-lactate in dairy products

Homogenized whole milk and yogurt were stored at room temperature (22–25°C) for 4 days and allowed to spoil. Spoiled milk and yogurt, and fresh yogurt samples were mixed by stirring with a spatula and centrifuged at 3200 × *g* (SORVAL RC-5) for 15 min to remove milk solids. The supernatants were then filtered through a 0.2-µm filter (Millipore, Bedford, MA) and the concentration of L-lactate measured using the biosensor and LOD enzyme assay.

The biosensor measurements were made with a LOD working electrode poised at +150 mV in 10 mL of 0.1 M, pH 7.0, phosphate buffer after constant residual current had been established. A suitable volume of the prepared sample was injected into the electrochemical cell and the increase in anodic current was recorded. The L-lactate amount was determined from a standard calibration curve prepared immediately prior to analysis.

L-lactate in milk and yogurt samples was also determined enzymatically by monitoring production of hydrogen peroxide formed during conversion of lactate to pyruvate in the presence of LOD. Hydrogen peroxide formation was monitored using the well established Trinder reaction in which H_2O_2 was reacted with phenol and 4-aminoantipyrine to form quinoneimine dye in the presence of horseradish peroxidase. The dye had a maximum absorption at 500 nm with a molar coefficient of 13000 $M^{-1}cm^{-1}$ (Bergmeyer, 1983). The reaction mixture contained 250 µL each of 3 mM phenol and 3 mM 4-aminoantipyrine, 2.5 U horseradish peroxidase, 300 µL of appropriately diluted filtered milk or yogurt sample and L-lactate oxidase in 0.1 M phosphate buffer (pH 7.0). The total volume was 930 µL. The blank composition was identical except for phosphate buffer in place of milk or yogurt.

RESULTS & DISCUSSION

Optimization of conditions

The response of a mediated-enzyme electrode is affected by several parameters. These include composition of the carbon paste matrix, operating potential, enzyme loading of the matrix, pH and temperature. The composition of the carbon paste, which contained graphite, mineral oil and TTF, was an optimum composition based on previous results (Almeida and Mulchandani, 1993, Gunasingham and Tan, 1990). These results had shown that high loading of TTF (> 20% w/w) decreased electrical conductivity of the paste. As in our previous work, a 13% (w/w) TTF loading was used in preparation of the LOD electrode.

Enzymes have been incorporated in carbon paste-mediated enzyme electrodes by either mixing them into the paste (Wang et al., 1990; Pandey et al., 1993) or by cross-linking the enzyme using glutaraldehyde on the top of the carbon paste and then retaining the cross-linked gel behind either a dialysis membrane (Gunasingham and Tan, 1990) or an electrochemically deposited polymer film (Almeida and Mulchandani, 1993). Mixing the enzyme into the carbon paste is a very simple technique. This, however, unnecessarily wastes a large quantity of enzyme, considering that only enzyme on the surface of the carbon paste is involved in catalysis of the reaction. It may therefore be efficient only for enzymes which are not costly and have high specific activity, such as glucose oxidase. The deposition of enzyme by cross-linking with glutaraldehyde is an effective technique for immobilizing a large quantity of enzyme just on the electrode surface. However, not all enzymes are amenable to glutaraldehyde treatment, as was observed during our attempts to immobilize LOD by that technique. LOD electrodes prepared by immobilizing enzyme by cross-linking and entrapment behind 1,3-phenylenediamine and resorcinol polymer films did not respond to lactate (data not shown). Attempts were made to immobilize the enzyme by adsorption. However, a very small amount of enzyme was adsorbed on this surface due to the highly hydrophobic nature of the oil-coated carbon particles. In order to overcome this problem the described surfactant treatment was adopted. The time of exposure to surfactant and procedure used had been previously optimized in our laboratory using glucose oxidase as test enzyme (details published elsewhere). By the surfactant treatment large quantities of enzyme could be adsorbed on the surface of the carbon paste. This was hypothesized as due to the decrease or elimination of oily insulating surface layer around the carbon particles at the surface.

The effect of operating potential on performance of the TTF-mediated carbon paste electrode has been previously investigated in our laboratory for a TTF-mediated glutamate biosensor (Almeida and Mulchandani, 1993). The potential of +150 mV found optimum for the glutamate electrode was selected for this study.

LOD in soluble form has a broad pH optimum, between 6.0 and 9.0. On immobilization by adsorption on carbon paste electrode the enzyme demonstrated a similar broad pH optimum but with maximum at pH 7.0 (Fig. 1). This pH optimum for the

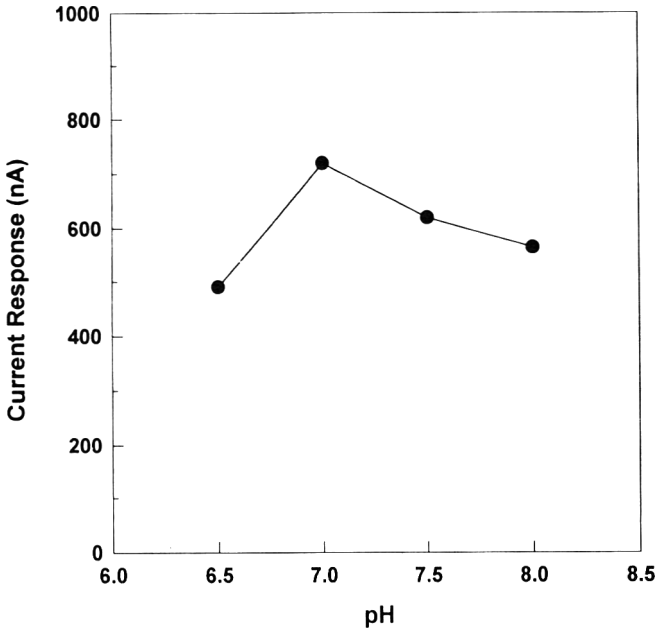


Fig. 1—Effect of solution pH on response of the biosensor to 1 mM L-lactate. Steady state currents measured at 25°C, +0.15 V vs Ag/AgCl and enzyme loading of 4.25 U LOD.

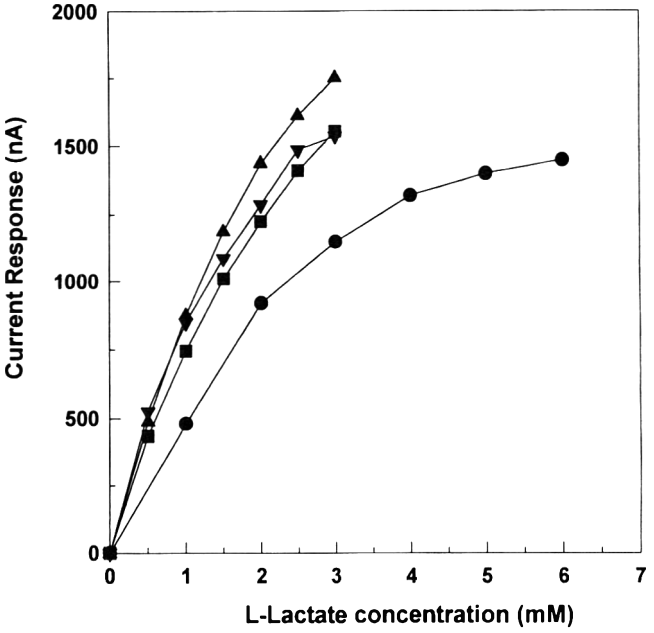


Fig. 2—Effect of temperature on response of biosensor to 1 mM L-lactate. Steady state currents at pH 6.5, +0.15 V vs. Ag/AgCl and enzyme loading of 4.25 U LOD. ● 20°C; ■ 25°C; ▲ 30°C; ▼ 37°C.

TTF-mediated biosensor was similar to that reported for a non-mediated lactate-oxidase electrode based on hydrogen peroxide detection that had been prepared by entrapment of lactate oxidase behind an electrochemical film (Dempsey et al., 1993). It was also similar to that for a methylene green-mediated carbon paste biosensor (Kulys et al., 1993). However, lactate enzyme electrodes configured by incorporating dimethyl ferrocene (DMFc) and Meldola blue (MaB) mediators in carbon pastes exhibited pH optima of 9.3 and 7.9, respectively (Kulys et al., 1992). This difference in optimum pH for different mediators may be due to different physico-chemical properties of the enzyme in their presence at the carbon paste surface.

The effect of temperature on biosensor response (Fig. 2) showed both the linear range and the sensitivity of the enzyme electrode increased with increase in temperature from 20 to 30°C. However, when the temperature was increased further to 37°C both linear range and sensitivity dropped. This could be due to deactivation of the LOD, which has a reported optimum of 35°C in soluble form, during electrode stabilization before testing. The enzyme electrodes were therefore operated at 30°C. The activation energy for LOD immobilized by adsorption on carbon paste, determined by plotting the output current as a function of inverse of the temperature (in Kelvin), was 10.53 kcal gmol $^{-1}$.

The effect of enzyme loading on the biosensor surface on the response of the electrode to lactate (Fig. 3), showed electrode response increased with an increase in LOD from 2.15 to 8.5 U and then decreased when loading increased further. This decrease in response was contrary to expectation because, according to enzyme kinetics, the response should have plateaued, or increased. We hypothesized that at higher enzyme loading (12.75 U) the presence of a large amount of nonconducting protein on the electrode surface may have adversely affected formation of the 1,3-phenylenediamine-resorcinol polymer film resulting in low entrapment of the enzyme. This may have therefore resulted in a loss of enzyme in subsequent washing. In subsequent studies an enzyme loading of 8.5U was used.

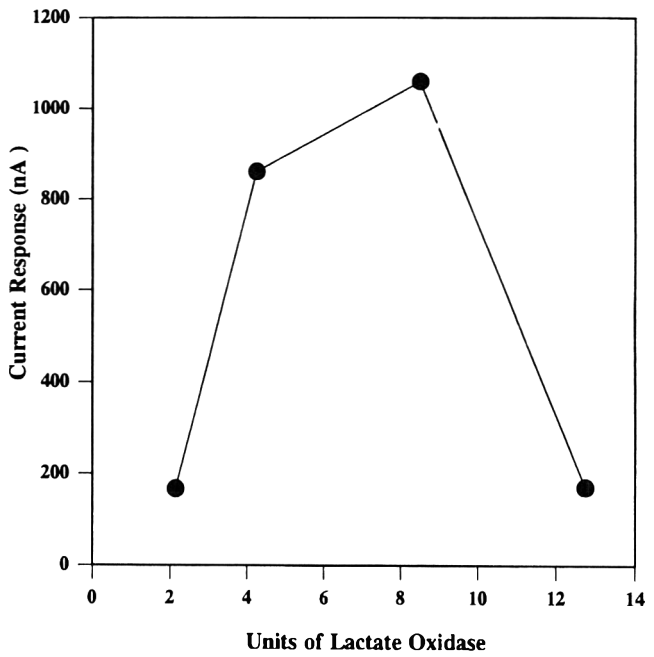


Fig. 3—Effect of enzyme loading on response of biosensor to 1 mM L-lactate. Steady state response at pH 7.0, 30°C and +0.15 V vs Ag/AgCl.

Analytical characteristics of the biosensor

Successive calibration plots for two identical lactate enzyme electrodes (Fig. 4) showed that the six calibration plots for the two electrodes could be divided into two distinct sets: (1) that for newly prepared electrodes (open and filled circles) and (2) that for electrodes used for the 2nd (open and filled squares) and 3rd (open and filled triangles) time. Analysis of the two sets of data revealed a decrease in sensitivity of the electrodes after the first calibration, presumably due to initial loss of TTF when the electrode was polarized for the first time. This was similar to results observed for the ferrocene-mediated enzyme electrode (Cass et al., 1984; Wang et al., 1990). Also, after an initial decrease of sensitivity the electrodes were very stable for a long time, which is desirable since this alleviates need for frequent electrode calibrations. Similarity of calibration plots for the two electrodes also demonstrated a low inter-electrode variability.

The calibration plot (prepared by pooling data of 2nd and 3rd calibrations for the two electrodes in Fig. 4) for the TTF-me-

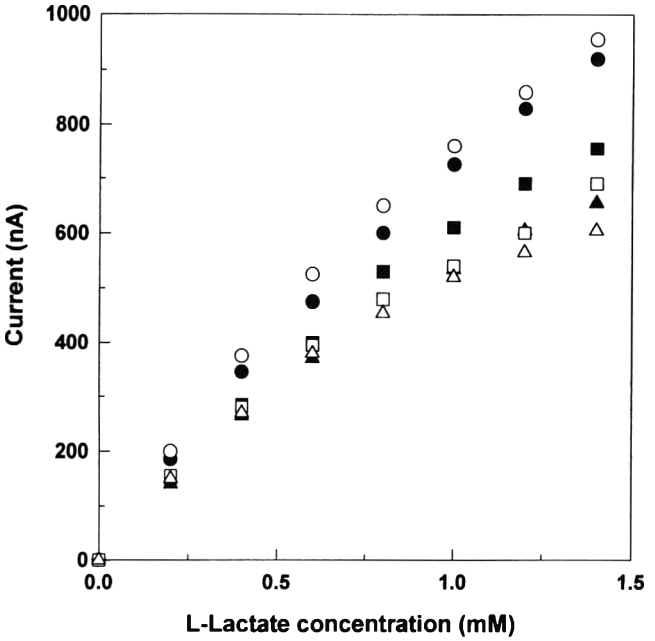


Fig. 4—Reproducibility of lactate biosensor calibrations. Steady state currents at pH 6.5, +0.15 V vs Ag/AgCl, 30°C, and enzyme loading 8.5 U LOD. ●, ○ 1st calibrations; ■, □ 2nd calibrations; ▲, △ 3rd calibrations. Open symbols: electrode 1; filled symbols: electrode 2.

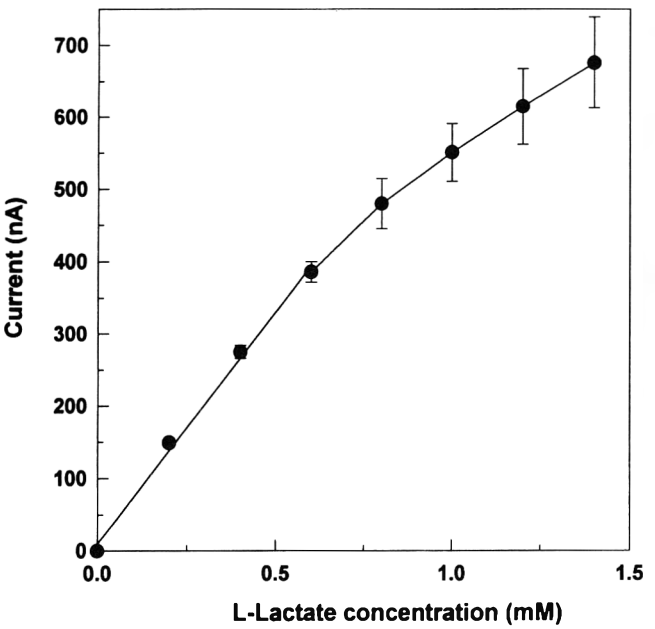


Fig. 5—Calibration plot for biosensor operating at pH 7.0, 30°C, +0.15 V vs Ag/AgCl and enzyme loading of 8.5 U LOD.

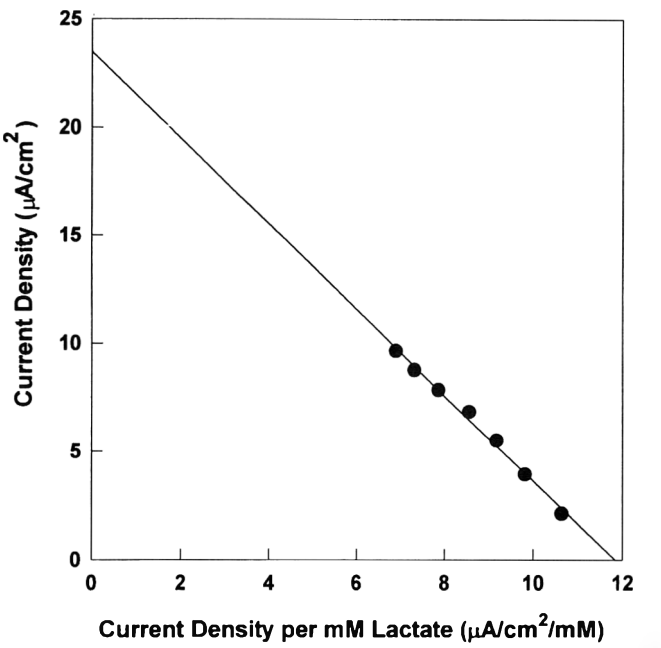


Fig. 6—Electrochemical Eadie-Hofstee plot for the biosensor operated at +0.15 V vs Ag/AgCl, 30°C and pH 7.0 and enzyme loading of 8.5 U LOD.

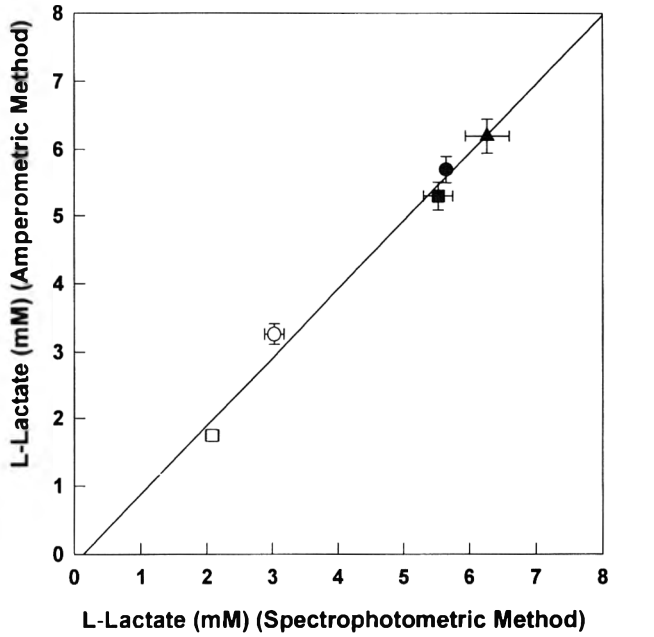


Fig. 7—Comparison of L-lactate measurement by the biosensor and enzyme assay technique. Results are averages of three measurements. □ Fresh commercial yogurt A; ○ Fresh commercial yogurt B; ■ Spoiled commercial yogurt A; ● Spoiled commercial yogurt B; ▲ Spoiled homogenized milk.

diated electrode showed response was linearly related to L-lactate concentration to 0.6 mM (Fig. 5). The calibration line had a slope of 642 nA mM^{-1} and a correlation coefficient of 0.9956. The minimum detection limit (defined as three times the standard deviation of the response of the electrode to injections of blank phosphate buffer) was found to be 56 μM (n=12). At each injection of L-lactate, a stable response was reached in ≈90 sec. Additionally, for unknown reason(s) we found that at higher lactate concentrations noise in the recorded signal increased and this resulted in increased error.

The current concentration dependence calibration graph over its whole range was like a Michaelis-Menten dependence. The apparent kinetics parameters j_{max} and K_m^{app} could be determined from the electrochemical Eadie-Hofstee form of the Michaelis-Menten equation (Hale et al., 1991; Almeida and Mulchandani, 1993):

$$j = j_{max} - K_m^{app} (j/C) \quad (4)$$

where j is the steady state current density, j_{max} is the maximum current density under conditions of enzyme saturation and C is the lactate concentration. The values of K_m^{app} and j_{max}, (from Fig. 6) were determined to be 1.98 mM and 23.47 μA cm^{-2}, respectively.

The long-term storage lifetime stability of the enzyme electrode was investigated by evaluating the response of the elec-

trode to lactate regularly and storing in back at 4°C in pH 7.0 phosphate buffer. The biosensor response was very stable (90% of the original) for about 36 days. It later decreased rapidly and it was completely unresponsive after 96 days.

Determination of L-lactate in dairy products

The lactate enzyme electrode operating at optimum conditions was used to determine lactate concentrations in dairy products and results were compared to those by conventional spectrophotometric enzymatic methods (Fig. 7). The high correlation (slope = 0.9972, correlation coefficient 0.9932) between the two methods indicated that the sensor was reliable for detecting L-lactate in milk and yogurt.

CONCLUSION

THE LACTATE BIOSENSOR provides advantages in rapidity of analysis and simplicity of operation compared to the spectrophotometric method and, therefore, can be used for routine, efficient and rapid monitoring of dairy products. Additionally, the electrode can be very easily adapted to a flow injection (FIA) system for on-line monitoring applications in food or biotechnology industries.

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enable development of procedures to measure calpain activity in cell extracts or biopsy-sized samples. The technique can enhance our understanding of the functions of the calpain enzymes and calpastatin in postmortem myofibrillar protein degradation and meat tenderization.

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Volatiles and Sensory Characteristics of Cooked Egg Yolk, White and Their Combinations

M.W. WARREN, D.K. LARICK, and H.R. BALL, JR.

ABSTRACT

Headspace volatiles were produced by heating fresh egg yolk, white and different ratios of yolk to white. Volatiles of the same treatments produced during steam distillation extraction were identified, compared, and related to sensory characteristics of fresh scrambled eggs. Overall impression, sulfur, and sweet notes were sensory characteristics identified to distinguish between samples with varying yolk:white ratios. Volatile concentrations of compounds in the headspace, and steam distillation/solvent extracts were used to distinguish between scrambled eggs with different yolk:white ratios. The concentration of both egg yolk and white had a significant effect on fresh scrambled egg sensory characteristics and flavor volatiles. The contribution of both must be considered when producing egg substitutes.

Key Words: egg yolk, egg white, scrambled eggs, egg flavor

INTRODUCTION

CONSUMER CONCERN about dietary cholesterol has affected consumption of eggs and egg products and many low-cholesterol egg substitutes have been developed. These products usually contain egg white, milk solids, vegetable oil and little or no egg yolk. In general, egg substitute products have been reported to be inferior in flavor and aroma to whole egg products in sensory evaluations (Leutzinger et al., 1977; Gardner et al., 1982; Warren and Davis, 1990). Differences in flavor attributes of such products have been associated with the absence of egg yolk components. Warren and Davis (1990) developed scrambled egg products that contained dried egg yolks, from which some fat and cholesterol had been removed. When such products were compared by consumers to commercial nonyolk scrambled egg products, partially defatted yolk products were equally preferred to non-yolk commercial products. Warren and Davis (1990) attributed this to flavor components provided by defatted yolks.

Researchers have attempted to identify volatile flavor components that contribute to egg's unique flavors and aromas. MacLeod and Cave (1975, 1976) used a simultaneous steam distillation-solvent extraction apparatus to separate 116 flavor volatiles from egg (≈65 identified). Umano et al. (1990) used a simultaneous purging-extraction apparatus in which egg was heated to 200°C for 1 hr. and 141 compounds were identified. Purge-and-trap systems in which volatiles are collected on a Tenax column and then desorbed onto a gas chromatography column have also been utilized (Rayner et al., 1980; Flanders et al., 1981, 1983; Brown et al., 1986; Matiella and Hsieh, 1991). These methods required less sample heating and fewer volatiles were produced. These may be more representative of egg flavor than extreme heat procedures which may produce an excess of volatiles. No specific compound or limited group of compounds have been defined as imparting egg flavor (Warren and Ball, 1991). This may be due to the lack of sensory analyses in combination with chemical analyses of egg products.

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Our objectives were to: (1) identify headspace volatiles produced by heating fresh egg yolk, white and different ratios of yolk to white; (2) identify volatiles produced from the same samples during a modified Likens-Nickerson steam distillation extraction; (3) evaluate the influence of fresh egg yolk, white, and different ratios on egg flavor; and (4) relate headspace and extracted volatile concentrations to sensory characteristics of fresh scrambled eggs.

MATERIALS & METHODS

Materials

Eggs from Single Comb White Leghorn laying hens, fed a typical corn-based diet, were used. Hens were 52 and 79 wk old for replication one, 54 and 81 wk old for replication two, and 56 and 83 wk old for replication three. Eggs (108/replication) were collected 1 wk before each sensory panel and stored, in plastic cartons, at 2° to 4°C. Eggs were 8 days old when presented to panelists.

Treatments of yolk:white ratios of 58:42%, 53:47%, 34:66% (control), 15:85% and 10:90% and 100% yolk and 100% white were prepared for sensory analysis as described by Warren and Ball (1991). Before blended eggs were cooked, 250 mL of liquid treatments were retained in sterile polyethylene bags at 2–4°C, for headspace gas chromatography and Likens-Nickerson volatile analyses. The pH of each liquid treatment and control was determined with a Fisher Accumet pH meter model 600 (Fisher Scientific, Springfield, NJ).

Sensory evaluation

Samples were prepared and served as described by Warren and Ball (1991). Aroma and flavor were evaluated by a pre-existing, 6-member, trained flavor profile panel (Caul, 1957; ASTM, 1981; Einstein, 1991) using a 14-point universal intensity scale (0 = not detectable, 14 = strongly detectable). During initial sessions, the panel trained specifically on egg samples of varying yolk:white ratios to develop a ballot of aroma and flavor descriptors, descriptor definitions (Warren and Ball, 1991) and to determine intensity levels in a 34% yolk:66% white reference standard. The reference sample (also included as a blind control) and other intensity references were available during each panel session. Panelists evaluated samples individually, then reached a concensus score. No more than four samples were evaluated during a session, therefore two sessions were required to complete each replication.

Quantitative headspace volatile analysis

Headspace analysis was performed on the same day as the flavor profile panel. Samples (2g) were weighed into a headspace vial which was sealed with Teflon-lined rubber septa. Samples were heated at 100°C for 30 min in a Perkin-Elmer HS-6 headspace sampler (Perkin-Elmer Corp., Norwalk, CT) and pressurized for 4 min. A standardized portion of headspace volatiles were automatically injected, for 1 min, onto a 30m DB-5 (J & W Scientific, Folsom, CA) capillary column with internal diameter 0.32 mm and a 1.0 μm film thickness. Carrier gas was ultra high purity (99.999% pure) helium. The oven was held at −30°C for 1 min and then programmed to 220°C at 4°C/min. The injector was 310°C and detector 320°C. A split flow of 31.0 mL/min with a column flow of 7.65 mL/min was used. Each sample was analyzed in duplicate. Data were collected and FID detector signals were integrated with a Waters 820 Chromatography Data Station (Millipore Waters Chromatography Division, Milford, MA). Peak areas for headspace volatiles were average areas of duplicates.

Quantitative steam distillation/solvent extraction

Egg flavor volatiles were extracted from heated eggs utilizing a modified Likens and Nickerson simultaneous steam distillation solvent extraction apparatus (Likens and Nickerson, 1964). The remaining liquid egg, retained from sensory analysis in polyethylene bags, was cooked in a stainless steel double boiler, without cooking aids, as described by Warren and Ball (1991). Scrambled egg (25g) was immediately weighed in a beaker and quantitatively transferred with 75 mL of distilled water into a blender and blended for 15 sec. Homogenized egg and water were quantitatively transferred to a 2L round bottom flask with 75 mL of distilled water. Samples were heated to 60°C and a magnetic stir bar was used to continuously agitate the egg mixture during heating. Extraction solvent, 25 mL of GC/MS grade hexane (Baxter Diagnostics, Inc., McGaw Park, IL), was poured into a 50-mL round bottom flask, containing glass boiling beads. Solvent was heated to 80°C. Egg was not heated until after the hexane began to distill. Total extraction time was 2 hr. After extraction was completed the hexane, containing extracted egg volatiles, was evaporated to 2–3 mL under vacuum at 25°C. Samples were quantitatively transferred to a 5 mL volumetric test tube with 2–3 mL of hexane and concentrated to 0.5 mL under a stream of prepurified (99.99% pure) nitrogen. Samples (0.5 µL) were injected onto the same column used for headspace analysis. The same conditions were used except the final temperature was 290°C. Peak areas for extraction analysis were averages of the three replicates. The remainder of the hexane samples were heat sealed in glass ampules and stored at −60°C until GC/MS analysis.

Qualitative analysis by gas chromatography/mass spectroscopy

Volatile components for headspace and extraction analysis were tentatively identified based on retention times of authentic standards and confirmed using a Hewlett Packard Model 5987 GC/MS with ionization potential 70 eV and scan range 30 to 300 m/e. Identification was achieved by comparing MS data with that of NIH/EPA reference spectra (NIH/EPA, 1978). Sample introduction included dynamic purge-and-trap sampling and direct injection.

Liquid whole egg for the purge-and-trap system was prepared as described by Warren and Ball (1991) except 30 eggs were used instead of 108. Eggs were from the same laying hens used in the sensory, headspace, and extraction experiments. Yolk and white were weighed and mixed to duplicate the control (34% yolk: 66% white) sample. The mixture was blended as described earlier (Warren and Ball, 1991). A 20 mL sample for purging was cooked in a covered, 25 mL needle sparge glass tube immersed in boiling water for 2 min. HPLC grade water (Fisher Scientific, Springfield, NJ) was added (10 mL) and mixed well with a stainless steel rod. The tube was attached to a Tekmar LSC-3 headspace concentrator (Tekmar Corp., Cincinnati, OH) and wrapped with heat tape. To eliminate foaming, samples were heated and purged as follows: heated to 50°C then purged 10 min, heated to 60°C then purged 10 min, heated to 80°C then purged 20 min, and heated to 95°C then purged 20 min for a total purge time 1 hr. Purging was with ultra high purity helium. Volatiles were collected on a 177mm × 6.3 mm Tenax trap (Anspec Co., Ann Arbor, MI) packed with Tenax TA (Alltech Associates Inc., Deerfield, IL). Volatiles were thermally desorbed, at 170°C, onto an identical capillary column as described above. Initial oven temperature (−30°C) was held 1 min after which the temperature was programmed to 220°C at 4°C/min.

For direct injection, extraction samples, that had been stored at −60°C, were quantitatively transferred to a 10 mL volumetric test tube with ≈8–10 mL of hexane, evaporated with prepurified nitrogen and transferred to a 5 mL volumetric test tube with ≈3–4 mL of hexane. Samples were concentrated to 0.5 mL. A 0.5 µL sample was then directly injected onto the capillary column and an identical temperature program was utilized.

Statistical analysis

Sensory profile evaluation, quantitative headspace and extraction analysis were replicated three times. For extraction, headspace, and pH data a randomized complete block design ANOVA procedure was utilized. Sensory data was analyzed by the randomized complete block design General Linear Models (GLM) regression procedure of SAS Institute, Inc; (1985). Waller-Duncan K ratio t-tests (SAS Institute, Inc., 1985) were computed for main effect means which were significantly different. Correlation coefficients (SAS Institute, Inc., 1985) between sensory and instrumental flavor measures and between pH and sensory characteristics were also computed. Stepwise discriminant analysis (SAS Institute, Inc., 1985) was used to select a subset of variables for classifying observations into groups. Models were selected using a stepwise discriminant selection procedure. At each step, the variable that contributed least to the discriminatory power of the model, as measured by Wilks' lambda, was removed from the model. Selection was complete when all variables in the model met criterion to stay and no remaining variables could be entered (SAS Institute, Inc., 1985). Observations were classified into treatments by computed discriminant functions. Canonical variables were derived and pairs were plotted to aid in visual interpretation of group differences (SAS Institute, Inc., 1985).

RESULTS & DISCUSSION

Headspace volatile analysis

A total of 60 volatiles were quantitated using headspace analysis of which 32 were identified (Table 1). They were grouped into 11 classes which included 12 aldehydes, 2 ketones, 1 alcohol, 5 alkanes, 3 sulfur containing compounds, 1 furan, 1 ether, 1 ester, 4 alkylbenzenes, 1 fatty acid and 1 chlorine containing compound (Table 1).

Those in greatest concentration in control samples were aldehydes (Table 1), with acetaldehyde highest. Acetaldehyde has been previously identified in whole eggs (MacLeod and Cave, 1976; Brown et al., 1986). The second most concentrated aldehyde was 3-methylbutanal. Matiella and Hsieh (1991) identified this compound as the most abundant in supermarket and freshly laid whole eggs. MacLeod and Cave (1976), utilizing steam distilled egg, reported acetaldehyde was the most abundant in egg yolk followed by 3-methylbutanal, hexanal, diethylether, 2-methylpropanal, acetone, heptane, pentanal, dimethyl disulfide, and then 2-methylbutanal. In our results acetaldehyde was in highest concentration in headspace volatiles of whole egg (control) with less found in both yolk and white (Table 1). Similarly, other aldehydes in highest concentration in the control, 3-methylbutanal, hexanal, and 2-methylbutanal decreased as yolk or white increased. The decrease in concentration was greater as egg white concentration increased and yolk concentration decreased.

Two ketones were identified, acetone and 3-methyl-2-butanone (Table 1). Acetone, has been reported in eggs (MacLeod and Cave, 1975, 1976; Brown et al., 1986; Umano et al., 1990). Umano et al. (1990) determined that acetone was present in whole egg and white but not yolk. We found it present in yolk and not in white. High concentrations of acetone were in the control and treatments with more yolk. White (100%) had only traces of acetone. 3-Methyl-2-butanone has not been previously reported in eggs. It was most abundant in egg yolk and decreased as yolk decreased and white increased. Methyl ketones have been implicated in off flavors referred to as "perfume" rancidity in some foods (Stokoe, 1928).

The only alcohol in headspace volatiles (Table 1) was octenol, and it was in very low concentrations. Heath and Reineccius (1986) indicated that alcohols usually make minor contributions to flavor in food systems. Five alkanes were identified. In their simultaneous steam distillation-solvent extracts, MacLeod and Cave (1976) identified all saturated straight chain hydrocarbons from C_7 to C_{17}, with n-pentadecane the major constituent. They suggested that these compounds were produced by decarboxylation of fatty acids from glycerides. Low molecular weight alkanes (C6 and C7) were in higher concentrations in control and yolk samples than in those containing more egg white (Table 1). Higher molecular weight alkanes (C13 and C14) were in higher concentrations in samples containing primarily egg white.

Three sulfur compounds were identified in headspace volatiles (Table 1). Dimethyl sulfide and dimethyl disulfide have been identified (MacLeod and Cave, 1975, 1976; Umano et al., 1990; Matiella and Hsieh, 1991). However, thiirane has not been noted in egg flavor. The highest concentration of dimethyl disulfide was in egg yolk (Table 1) and it tended to decrease as white was added. Umano et al. (1990) reported that the concentration

Table 1—Relative concentrations (peak areas, mV-sec) of headspace volatile compounds identified[y] in scrambled fresh whole egg, egg yolk, egg white, and different ratios of yolk to white

Peak No.	Compound name by class	Identification[z]	Average peak areas (mV-sec) Yolk	58:42	53:47	Yolk:White Ratio Control 34:66	15:85	10:90	White
			100						100
	Aldehydes								
5	acetaldehyde	(MS)	37,100^{e}	52,472^{cd}	65,859^{ab}	71.198^{a}	60,300^{bc}	49,515^{d}	20,769^{f}
14	2-methylpropanal	(MS,RT)	4,759^{d}	5,657^{cd}	6,708^{c}	10.756^{a}	8,474^{b}	5,213^{cd}	266^{e}
17	butanal	(RT)	377^{cd}	978^{b}	2,184^{a}	2 067^{a}	469^{c}	260^{cd}	16^{d}
23	3-methylbutanal	(MS,RT)	24,258^{c}	25,663^{bc}	30,160^{b}	38.815^{a}	26,772^{bc}	17,754^{d}	15^{e}
24	2-methylbutanal	(MS)	2,083^{c}	2,637^{c}	2,812^{c}	5 181^{a}	4,078^{b}	2,529^{c}	67^{d}
25	pentanal	(MS,RT)	3,344^{c}	7,376^{a}	5,393^{b}	6 336^{ab}	860^{d}	110^{d}	21^{d}
30	hexanal	(MS,RT)	13,592^{c}	23,298^{b}	25,504^{ab}	29 161^{a}	8,836^{d}	3,901^{e}	13^{e}
34	heptanal	(MS,RT)	394^{c}	1,022^{b}	1,177^{b}	1 545^{a}	467^{c}	173^{cd}	17^{d}
39	octanal	(MS,RT)	168^{b}	767^{a}	610^{a}	780^{a}	156^{b}	144^{b}	92^{b}
41	nonanal	(MS,RT)	207^{d}	584^{bc}	789^{ab}	1 080^{a}	169^{d}	78^{d}	254^{cd}
42	t-2 nonenal	(RT)	16^{e}	35^{bc}	59^{ab}	87^{a}	65^{ab}	65^{ab}	86^{e}
43	2,4-decadienal	(MS,RT)	265^{e}	674^{d}	791^{d}	1,025^{c}	1,343^{b}	1,472^{ab}	1,599^{a}
	Ketones								
10	acetone	(MS)	4,403^{cd}	11,274^{b}	14,370^{b}	18 125^{a}	7,099^{c}	1,545^{de}	24^{e}
16	3-methyl-2-butanone	(RT)	1,865^{a}	897^{b}	860^{b}	564^{c}	307^{d}	314^{d}	154^{d}
	Alcohols								
37	octenol	(MS,RT)	58^{cde}	153^{ab}	98^{bcd}	175^{a}	110^{abc}	26de	19^{e}
	Alkanes								
18	hexane	(MS)	267^{b}	729^{a}	778^{a}	818^{a}	216^{b}	135^{b}	9^{b}
22	methylcyclopentane	(MS)	223^{bcd}	358^{abc}	514^{ab}	544^{a}	418^{abc}	189^{cd}	37^{d}
26	heptane	(MS,RT)	4,338^{bc}	6,693^{ab}	8,128^{a}	7,256^{a}	2,632^{cd}	627^{d}	24^{d}
44	tridecane	(RT)	23^{a}	68^{d}	59de	119^{c}	173^{b}	224^{a}	229^{a}
45	tetradecane	(RT)	12^{e}	50de	52^{d}	74^{cd}	102^{c}	164^{b}	238^{a}
	Sulfur								
12	dimethyl sulfide	(MS)	897^{a}	940^{a}	998^{a}	915^{a}	846^{a}	443^{b}	7^{c}
15	thiirane	(MS)	266^{bc}	288^{bc}	184^{cd}	550^{a}	470^{ab}	242^{c}	21^{d}
27	dimethyl disulfide	(MS)	2,343^{a}	686^{b}	809^{b}	389^{c}	314^{cd}	222^{cd}	104^{d}
	Furans								
38	2-pentyl furan	(MS)	1,354^{b}	1,500^{ab}	1,709^{a}	1,740^{a}	438^{c}	233^{cd}	113^{d}
	Ethers								
11	diethylether	(MS)	6,395^{ab}	7,066^{a}	6,814^{a}	6,500^{a}	6,653^{a}	5,185^{b}	1,559^{c}
	Esters								
21	ethylacetate	(MS)	300^{bc}	278^{bc}	529^{b}	1,387^{a}	511^{b}	208^{bc}	19^{c}
	Aromatics								
28	toluene	(MS)	133	138	138	124	179	119	105
33	1,2-dimethylbenzene	(MS)	40	117	148	144	110	95	67
35	benzaldehyde	(MS)	513^{b}	852^{a}	964^{a}	1,154^{a}	427^{b}	211^{bc}	24^{c}
36	trimethylbenzene	(RT)	329^{ab}	303^{ab}	263^{ab}	413^{a}	208^{bc}	190^{bc}	79^{c}
	Fatty Acids								
40	heptanoic acid	(MS,RT)	137	314	323	337	471	526	359
	Chlorine								
20	chloroform	(MS)	246^{e}	282^{a}	239^{a}	247^{a}	277^{a}	225^{a}	17^{b}

[a-f] Values in the same row with different superscripts differ significantly ($P<0.01$).

[y] Only those peaks identified are presented.

[z] MS=identified from interpretation of mass spectroscopy (complete spectrum) data; RT=data consistent with that of authentic compounds.

of dimethyl disulfide was highest in whole egg, then yolk and lowest in egg white. In our results, dimethyl sulfide was also in lowest concentration in egg white (Table 1). Thirrane was more concentrated in the control and 15:85 yolk:white ratio treatment and least concentrated in 100% white (Table 1). Flanders et al. (1983) isolated 16 sulfur compounds from heated albumen and 13 from egg yolk. They noted that greater quantities of the principal components were produced by yolk. However, only hydrogen sulfide, ethanethiol, dimethyl sulfide and dimethyl disulfide were positively identified and methional and chlorobenzothiazole were tentatively identified.

Dimethyl disulfide forms by Strecker degradation of methionine and dimethyl sulfide is derived directly from dimethyl disulfide via free radical reactions (Schutte, 1974). Methionine content (0.39g/100g egg) in liquid yolk is the same as that for liquid whole egg and liquid white (Cook and Briggs, 1986). Thus, treatments should yield equal amounts of dimethyl disulfide, if equal amounts of egg solids were heated. In our results egg white contained fewer sulfur compounds than yolk. This may suggest that sulfur containing compounds in white were more easily decomposed during heating. The greater heat liability may be the result of differences in forms of sulfur, extent of protection of sulfur bearing amino acids in egg white proteins vs yolk proteins, or differences in pH between egg white (pH 9.0) and egg yolk (pH 6.1).

One alkylfuran, (2-pentyl furan), was identified in the headspace volatiles (Table 1). Matiella and Hsieh (1991) and Umano et al. (1990) also reported 2-pentyl furan in egg. 2-Pentyl furan was more concentrated in the control and treatments containing more yolk than in those treatments containing more white (Table 1). Diethyl ether was in fairly high concentrations in all samples containing at least 10% yolk (Table 1). Ethers of lower molecular weight are very volatile and have sweet, spirit-like odors while higher molecular weight ethers have ethereal and fruity odors (Heath and Reineccius, 1986). Ethyl acetate was the only ester identified in the headspace volatiles (Table 1).

Four aromatic compounds were identified in the headspace volatiles (Table 1). MacLeod and Cave (1975) reported that toluene was the major component in a series of alkylbenzenes ranging from benzene to n-octylbenzene. Benzaldehyde was the most abundant aromatic compound with whole egg containing its highest concentrations. Trimethylbenzene was the second most abundant compound in this class. Aromatics were more concentrated in treatments containing ratios of yolk:white that

were close to whole egg and were lowest in 100% white (Table 1). MacLeod and Cave (1975) suggested that aromatic hydrocarbons could form by thermal degradation of glucose or through an alternative pathway which would involve thermal degradation of carotenoids (MacLeod and Cave, 1975).

For headspace volatiles there was, in most cases, a larger ($P < 0.01$) amount of each compound in the whole egg and, as yolk or white increased or decreased, concentrations of the volatiles decreased. Exceptions to this trend were 2,4-decadienal and tetradecane which were in higher concentrations (Table 1) in samples with more egg white and 3-methyl-2-butanone and dimethyl disulfide which were in higher concentrations in samples containing more yolk. Diethyl ether was not different among treatments with more yolk, but the two highest white containing treatments contained less diethyl ether.

Extraction volatile analysis

Identified volatiles extracted with the Likens and Nickerson (1964) apparatus were compared (Table 2). Volatiles (33) were grouped into 8 classes which included 10 aldehydes, 2 ketones, 5 alcohols, 2 alkanes, 1 furan, 1 pyrazine, 6 aromatic compounds and 6 fatty acids. As expected, this extraction method isolated more high molecular weight compounds than the headspace volatile procedure. The aldehydes hexanal, heptanal, nonanal, and 2,4-decadienal had been identified utilizing headspace analysis; however, 2-octenal, 17-octadecenal, n-octadecenal and 9-octadecenal were only in the extracted volatiles. These aldehydes typically result from lipid oxidation. As a result, there were higher ($P < 0.01$) concentrations of these aldehydes in egg yolk and treatments that contained more yolk than in treatments that contained more white (Table 2). Heptanal was the only aldehyde which was more concentrated in whole egg than in egg yolk.

Two ketones were identified in extracted volatiles (Table 2). The presence of 5-methyl, 5-phenyl-2-hexanone in eggs has not been reported and its origin is not known. Alcohols in the extracted volatiles were higher in molecular weight and more abundant that those from headspace analyses. 2-Ethyl-1-hexanol has a mild, oily, sweet, slightly floral odor reminiscent of roses and a sweet, fatty floral taste (Furia and Bellanca, 1975). 2-Ethyl-1-hexanol was the most abundant alcohol in extracted samples.

Fewer alkanes were present in extracted volatiles (Table 2) as compared to headspace volatiles (Table 1). No differences occurred among treatments for tridecane or tetradecane. 2,5-Dimethyltetrahydrofuran (trans) was the only furan identified in extracted volatiles (Table 2). MacLeod and Cave (1975, 1976) identified this compound in whole eggs and egg yolk and described it as having a pungent odor.

One pyrazine (2,5-dimethylpyrazine) was identified in extracted volatiles and no differences occurred in this compound among treatments (Table 2). Umano et al. (1990) identified this pyrazine along with 15 others in egg heated to 200°C. MacLeod and Cave (1975, 1976) also isolated several pyrazines in distilled egg samples. Among these was 2,6-dimethylpyrazine which had an odor description of "burnt." Pyrazines are the major volatile flavor compounds produced in Maillard browning reactions, and they contribute roasted or toasted flavors to cooked foods (Umano et al., 1990). The presence of these pyrazines in egg volatiles extracted by steam-distillation or by heating to 200°C and purging (Umano et al., 1990) was likely due to the extreme heat which yielded higher molecular weight volatiles. Therefore, some of the volatiles (such as pyrazine) may not normally be obtained from typical cooked eggs (Umano et al., 1990).

Six aromatic compounds were identified from the extracted egg volatiles (Table 2). Unlike the headspace volatiles, benzaldehyde was not the major compound in this class. Benzacetaldehyde was in highest concentration and was the second most abundant compound in whole egg, as determined by extraction. 2-Ionol was in high concentration in all samples containing at least 42% egg white. Another compound concentrated in egg white was 1,2-benzene dicarboxylic acid. The concentration of aromatics was influenced by sample composition but no consistent trend was found related to content of yolk or white.

Sensory profile panel analysis

Flavor profiling results for aroma and flavor of the different treatments were compared (Table 3). Whole egg received higher intensity scores for overall impression, fresh scrambled egg flavor, and fresh scrambled egg aftertaste than did any of the 6 treatments (Table 3). As yolk or white ratios were adjusted there was a decrease in flavor and aroma intensity scores for fresh scrambled egg flavor, overall impression and fresh scrambled egg aftertaste. These results followed the trend of headspace volatiles. As yolk or white was adjusted, the concentration of volatiles decreased.

The pH of each of the treatments was also included (Table 3). As expected, pH of the treatments increased ($P < 0.01$) with increased proportions of egg white. Simple correlation coefficients between pH of the treatments and sensory profile panel scores for each aroma and flavor characteristic were calculated (Table 4). The overall impression, fresh scrambled egg and fresh scrambled egg aftertaste character notes for aroma and flavor were not significantly correlated with pH (Table 4).

As the ratio of white increased, an increase in intensity values for sulfur aroma and sulfur flavor occurred (Table 3). These results were consistent with those of Warren and Ball (1991). Sulfur aroma and flavor notes also positively correlated with pH (Table 4). Germs (1973) found that with rising pH, egg white produced increasing amounts of H_2S. In this case, the rise in pH was due to reduced amounts of yolk and increased egg white.

Three treatments; 100% yolk, 58:42, and 53:47 (yolk:white, respectively) had a sweeter aroma ($P < 0.01$) than whole egg and treatments with higher percentages of white (Table 3). This trend was not as clear for sweet flavor. The 100% yolk and 58:42 ratio samples were sweeter in flavor than 100% white and 10:90 ratio samples (yolk:white, respectively). Warren and Ball (1991) found that as yolk increased, an increase in intensity values occurred for sweet flavor. Sweet aroma and flavor negatively correlated with pH of treatments (Table 4).

The three treatments containing higher concentrations of yolk had higher ($P < 0.01$) intensity values for dairy aroma than the three treatments with higher concentrations of white (Table 3). A similar trend was observed for dairy flavor. Dairy flavor and aroma were negatively correlated with pH (Table 4). Sour aroma tended to increase as white content increased with the 90% white sample receiving the highest score (Table 3). Sour aroma positively correlated with pH (Table 4). Warren and Ball (1991) reported no differences, in sour aroma, among treatments with different yolk and white ratios. Astringent mouthfeel differed among treatments (Table 3). As either yolk or white increased, an increase in astringent mouthfeel occured. Astringent mouthfeel did not correlate with pH (Table 4). As egg white content decreased, there was a decrease in noseburn aroma (Table 3). Warren and Ball (1991) suggested that this "chemical" noseburn may be due to ammonia and hydrogen sulfide formed from heating proteins in egg white. Noseburn positively correlated with pH (Table 4). Germs (1973) reported that production of H_2S in heated egg increased with increasing temperature and pH and that ammonia production accompanied H_2S production on heating of egg whites.

Relationship between sensory and instrumental analyses

Stepwise discriminant analysis (SDA) could be used to select a subset of quantitative variables that best reveal differences among treatments. The sensory flavor characteristics of scrambled egg, selected by SDA to classify observations into treat-

Table 2—Relative concentrations (peak area, mV·sec) of extracted volatile compounds identified[y] in scrambled fresh whole egg, egg yolk, egg white, and different ratios of yolk to white

Peak no.	Compound name by class	Identification[z]	Average peak areas (mV-sec) Yolk 100	Yolk:White Ratio Control 58:42	53:47	34:66	15:85	10:90	White 100
	Aldehydes								
3	hexanal	(MS,RT)	1,009	1,660	1,363	508	360	282	175
6	heptanal	(MS,RT)	62^{B}	376^{B}	$1,254^{A}$	237^{B}	88^{B}	104^{B}	88^{B}
23	2-octenal	(MS)	$3,133^{a}$	$1,995^{ab}$	$1,649^{bc}$	698^{cd}	240^{d}	72^{d}	29^{d}
27	nonanal	(MS,RT)	3,183	4,430	4,959	2,592	2,401	2,586	2,529
36	2,4-decadienal (cis,trans)	(MS)	$2,073^{a}$	$1,869^{a}$	$1,677^{ab}$	563^{bc}	58^{c}	43^{c}	55^{c}
38	2,4-decadienal (cis,cis)	(MS)	$17,251^{a}$	$9,036^{b}$	$7,942^{b}$	$2,410^{c}$	322^{c}	198^{c}	9^{c}
55	17-octadecenal	(MS)	$253,212^{a}$	$109,025^{b}$	$114,419^{b}$	$71,076^{c}$	$33,495^{d}$	$23,307^{de}$	$1,882^{e}$
59	n-octadecenal	(MS)	$3,523^{a}$	$2,332^{b}$	$2,587^{b}$	$2,233^{b}$	$1,536^{c}$	$1,195^{c}$	92^{d}
62	9-octadecenal	(MS)	$25,932^{a}$	$11,890^{b}$	$13,363^{b}$	$7,957^{c}$	$3,996^{d}$	$2,984^{de}$	183^{e}
65	n-octadecenal	(MS)	$125,159^{a}$	$51,957^{b}$	$59,097^{b}$	$33,204^{c}$	$15,078^{d}$	$11,110^{d}$	$1,720^{d}$
	Ketones								
8	2,5-hexanedione	(MS)	119	420	430	5,124	972	88	464
50	5-methyl,5-phenyl 2-hexanone	(MS)	61^{d}	735^{c}	$1,499^{a}$	$1,178^{b}$	156^{d}	55^{d}	74^{d}
	Alcohols								
2	2-methyl-1-penten-3-ol	(MS)	39	359	145	301	215	85	121
14	octenol	(MS)	$2,685^{a}$	$1,841^{bc}$	$1,757^{ab}$	905^{cd}	456^{d}	528^{d}	205^{d}
19	2-ethyl-1-hexanol	(MS)	60,769	119,261	67,847	141,574	100,803	70,947	12,509
56	1-hexadecanol	(MS,RT)	$11,483^{ab}$	$11,675^{ab}$	$13,130^{a}$	$12,614^{ab}$	$9,210^{bc}$	$7,454^{c}$	$2,229^{d}$
67	1-octadecanol	(MS)	$1,609^{a}$	$1,120^{ab}$	$1,170^{ab}$	$1,692^{a}$	857^{bc}	731^{bc}	294^{c}
	Alkanes								
37	tridecane	(RT)	84	335	89	105	66	41	73
43	tetradecane	(RT)	367	522	525	611	356	356	419
	Furans								
1	2,5-dimethyl tetrahydrofuran (Z)	(MS)	267^{AB}	323^{A}	234^{AB}	67^{B}	132^{AB}	113^{AB}	51^{B}
	Pyrazines								
5	2,5-dimethylpyrazine	(MS)	449	820	764	505	321	310	102
	Aromatics								
10	benzaldehyde	(MS)	540	678	547	473	149	134	148
16	2-hydroxy benzaldehyde	(MS)	684^{b}	$1,183^{b}$	$1,083^{b}$	$2,792^{a}$	$1,083^{b}$	408^{b}	253^{b}
21	benzacetaldehyde	(MS)	$94,478^{c}$	$201,001^{b}$	$251,713^{a}$	$90,448^{c}$	$9,577^{d}$	$3,518^{d}$	573^{d}
30	naphthalene	(MS)	284^{c}	849^{AB}	879^{AB}	900^{A}	587^{ABC}	347^{C}	408^{BC}
49	2-ionol	(MS)	$6,214^{d}$	$16,416^{c}$	$19,180^{bc}$	$21,977^{bc}$	$27,539^{ab}$	$36,218^{a}$	$15,013^{cd}$
88	1,2-benzene dicarboxylic acid	(MS)	$28,478^{AB}$	$7,136^{B}$	$7,613^{B}$	$49,092^{AB}$	$4,788^{B}$	$11,146^{B}$	$95,566^{A}$
	Fatty Acids								
39	hexylester butanoic acid	(MS)	$1,277^{c}$	$6,618^{ab}$	$8,198^{a}$	$2,190^{bc}$	198^{c}	126^{c}	14^{c}
42	octylester butanoic acid	(MS)	608	931	860	986	633	645	622
61	hexadecanoic acid	(MS)	$8,807^{bc}$	763^{c}	$3,838^{bc}$	$13,342^{bc}$	$27,920^{b}$	$62,013^{a}$	$3,541^{c}$
71	9,12-octadecadienoic acid (trans,trans)	(MS)	309^{B}	80^{B}	267^{B}	538^{B}	$1,091^{AB}$	$2,267^{A}$	119^{B}
72	9-octadecenoic acid	(MS)	$2,567^{b}$	777^{b}	$2,313^{b}$	$5,451^{b}$	$8,712^{ab}$	$15,741^{a}$	117^{b}
74	octadecanoic acid	(MS)	706^{B}	92^{B}	169^{B}	$1,056^{B}$	$3,092^{AB}$	$6,549^{A}$	127^{B}

[a-f] Values in the same row with different superscripts differ significantly ($P < 0.01$).

[A-E] Values in the same row with different superscripts differ significantly ($P < 0.05$).

[y] Only those peaks identified are presented.

[z] MS = identified from interpretation of mass spectroscopy (complete spectrum) data; data; RT = data consistent with that of authentic compounds.

ments (concentration of egg yolk and egg white) were overall impression, sulfur, and sweet. Sweet was the only aroma note selected. The average squared canonical correlation (ASCC) was 0.4159; ASCC is close to 1.0 if all observations are well separated and if all or most directions in the discriminant space show good separation, for at least 2 groups (SAS Institute, Inc., 1985). Results of SDA (SAS Institute, Inc., 1985) indicated that there were observable and reproducible differences among sensory characteristics in the model since 100% of samples were correctly classified into the seven different ratios of yolk to white. The statistical rationale for selection of these characteristics appeared clear in that overall impression had higher ($P < 0.01$) intensity scores in whole egg (34% yolk:66% white) while sulfur flavor and sweet aroma and flavor had higher ($P < 0.01$) intensity scores for treatments with more egg white and more egg yolk, respectively (Table 3).

Peaks from headspace analysis which correlated ($P < 0.05$) to sensory parameters (Warren, 1991) were used in the stepwise selection procedure of SDA to classify samples into appropriate concentrations of egg yolk and white. Those compounds selected into the model included acetone, dimethyl disulfide, diethylether, 2-methylpropanal, methylcyclopentane, hexanal, 1,2-dimethylbenzene, trimethylbenzene, and unknown 7. The model selected for separating observations into the seven different treatments had an ASCC of 0.6888. Results of the SDA (SAS Institute, Inc., 1985) indicated that observable and reproducible differences among the 9 volatiles since 100% of samples were classified correctly. For the majority of volatiles selected (except 1,2-dimethylbenzene), the concentration of each was highest in whole egg, lower in treatments with higher yolk and in lowest concentrations in those with higher egg white (Table 1). All volatiles negatively correlated with pH and the sulfur, noseburn,

Table 3—pH and flavor profiling results for aroma and flavor of whole egg, egg yolk, egg white and different ratios of yolk to white

	Yolk	Yolk: White Ratio Control					White
	100	58:42	53:47	34:66	15:85	10:90	100
pH	6.20^{e}	7.03^{d}	6.97^{d}	7.43^{c}	8.23^{b}	8.40^{b}	9.07^{a}
Aroma							
Overall impression	3.32^{d}	4.24^{c}	5.04^{b}	6.45^{a}	5.27^{b}	4.66^{bc}	2.89^{d}
Scrambled fresh egg	2.66^{de}	3.09^{cd}	3.59^{b}	4.54^{a}	3.72^{b}	3.44^{bc}	2.53^{e}
Sulfur	1.33^{d}	1.87^{d}	1.94^{d}	2.76^{c}	3.32^{bc}	3.44^{b}	4.39^{a}
Sweet	2.71^{a}	2.79^{a}	2.60^{a}	2.09^{b}	2.06^{b}	2.00^{b}	1.95^{b}
Dairy	2.16^{ab}	2.26^{a}	2.26^{a}	1.98^{abc}	1.75^{c}	1.76^{bc}	1.25^{d}
Sour	1.71^{b}	1.63^{b}	1.84^{ab}	1.93^{ab}	1.99^{ab}	2.22^{a}	2.10^{ab}
Noseburn	2.04^{bc}	1.82^{c}	1.98^{bc}	2.00^{bc}	2.07^{bc}	2.34^{ab}	2.70^{a}
Flavor							
Overall impression	3.14^{d}	4.32^{c}	5.24^{b}	6.58^{a}	5.54^{b}	4.63^{c}	2.81^{d}
Scrambled fresh egg	2.81^{d}	3.26^{bc}	3.69^{b}	4.68^{a}	3.89^{b}	3.52^{bc}	2.32^{e}
Sulfur	1.44^{e}	1.76^{de}	1.99^{d}	2.21^{cb}	2.50^{c}	3.21^{b}	4.05^{a}
Sweet	2.55^{AB}	2.67^{A}	2.33^{ABC}	2.29^{ABC}	2.15^{ABC}	1.91^{C}	1.98^{BC}
Astringent mouthfeel	3.10^{AB}	3.00^{AB}	2.74^{B}	2.74^{B}	2.82^{B}	3.09^{AB}	3.45^{A}
Dairy	2.12^{a}	2.13^{a}	2.01^{ab}	1.94^{abc}	1.71^{bc}	1.63^{c}	1.17^{d}
Aftertaste	2.45^{d}	2.92^{c}	3.53^{b}	4.41^{a}	3.47^{b}	2.97^{c}	2.23^{d}

$^{a-c}$ Values in the same raw with different superscripts differ significantly ($P < 0.01$).
$^{A-C}$ Values in the same row with different superscripts differ significantly ($P < 0.05$).

Table 4—Correlation of pH with flavor profile results for aroma and flavor

Sensory Characteristic	
Aroma	
Overall impression	−.0738
Scrambled fresh egg	−.0318
Sulfur	.9254**
Sweet	−.7692**
Dairy	−.7719**
Sour	.6053**
Noseburn	.5613**
Flavor	
Overall impression	−.0543
Scrambled fresh egg	−.1405
Sulfur	.8992**
Sweet	−.6364**
Astringent mouthfeel	.2845
Dairy	−.8205**
Aftertaste	−.1426

** Significant at $P < 0.01$ leve .

and astringent mouthfeel aroma and flavor characteristics and positively correlated with sweet and dairy aroma and flavor (Warren, 1991). Thus, the aroma and flavor characteristics that had higher intensity scores in yolk (Table 3) positively correlated to the majority of volatiles selected by SDA.

Peaks from extraction analysis which correlated ($P < 0.05$) with sensory parameters (Warren, 1991) were used to select a model to predict concentration of egg yolk and white in scrambled egg. Stepwise selection of SDA resulted in a model with 20 volatiles (ASCC 1.0). They included; 2,5-dimethyltetrahydrofuran, hexanal, benzacetaldehyde, 2,4-decadienal, hexylester butanoic acid, 2-ionol, 17-octadecenal, n-octadecenal, 9-octadecenal, and 11 unknowns (14-18, 25, 29, 36, 40, 53, and 54). Results of SDA (SAS, 1985) indicated observable and reproducible differences among the 20 volatiles since 100% of the samples were classified correctly. The four higher molecular weight aldehydes chosen by SDA correlated negatively with pH and sulfur and noseburn aroma and flavor (Warren, 1991). These same aldehydes correlated positively with sweet and dairy aroma and flavor (Warren, 1991). Thus, these aldehydes positively correlated with sensory terms that best described yolk flavor (Table 3). Hexanal, 2,5-dimethyl-tetrahydrofuran, benzacetaldehyde and hexylesterbutanoic acid tended to follow similar trends as higher molecular weight aldehydes (Warren, 1991). The alcohol, 2-ionol however, did not. This compound positively correlated with pH and correlated negatively with sweet aroma and flavor (Warren, 1991).

CONCLUSIONS

THE CONCENTRATION OF EGG YOLK and white had a significant effect on fresh scrambled egg sensory characteristics and flavor volatiles produced upon heating. Overall impression, sulfur, and sweet flavor could be used to describe scrambled egg containing varying concentrations of yolk and white. In addition, headspace and steam distillation volatile analysis could distinguish between scrambled eggs with different yolk:white ratios and also could identify volatiles related to specific sensory characteristics. Several aldehydes, aromatic compounds, and dimethyl sulfide were selected, from headspace and extraction analysis by SDA to identify differences in scrambled eggs with varying ratios of yolk to white. Most of the volatiles selected by SDA correlated positively with sweet and dairy sensory characteristics which had higher intensity values in treatments with more egg yolk. Also these volatiles correlated negatively with sulfur sensory characteristics which had higher intensity values in treatments with more egg white.

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Dietary Menhaden Oil Influences Sensory Characteristics and Headspace Volatiles of Shell Eggs

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ABSTRACT

Eggs from hens fed n-3 fatty acids (n-3FA) may provide a means for increased marketing of these healthful fatty acids. Eggs from hens fed graded levels (0, 0.5, 1.5, 3.0%) of menhaden oil (MO) were evaluated for fatty acid composition, sensory characteristics, and headspace volatiles. Eggs from hens fed 1.5% and 3.0% MO contained the greatest n-3 FA content and were not different. A linear increase in fishy notes occurred as level of dietary MO increased from 0 to 1.5%; however, these notes were not different between 1.5% and 3.0% eggs. Changes in headspace volatile profiles were quantitative. No unique volatiles characterized eggs from hens fed MO; however, concentration differences were noted between eggs from hens fed all levels of dietary MO. Changes in volatile profiles in response to dietary MO may be responsible for perception of fishy notes in such eggs.

Key Words: unsaturated fatty acids, menhaden oil, chicken eggs, acceptability

INTRODUCTION

DIETARY RECOMMENDATIONS encourage consumption of at least 1-2 fish meals per week as clinical and epidemiological evidence suggest that as a result the risk of coronary heart disease may be reduced as much as 50% (Bonna et al., 1992; Drevon, 1992; Kromhout, 1992). A precedent has been established for inclusion of n-3 fatty acid, docosahexaenoic (22:6n-3); DHA), in the diet of pre-term infants to support development of brain and retinal tissues (Nettleton, 1993). Marine products are the only abundant source of the 20-carbon n-3 fatty acids, eicosapentaenoic (20:5n-3, EPA) and DHA, which are proposed to have the most biological effect (Harris, 1989). Seasonal availability, cost, and consumer preference may limit fish consumption, thereby excluding the primary source of 20-carbon n-3 fatty acids. Egg yolk n-3 fatty acid content has been readily modified by incorporation of fish oil and fish meal in the laying hen diet (Adams et al., 1989; Hargis et al., 1991; Van Elswyk et al., 1992). N-3 fatty acid-enriched shell eggs may provide a viable alternative source of these fatty acids, both independently and when included in further processed products. Additionally, the production of n-3 fatty acid enriched shell eggs may provide an alternative to largely unsuccessful attempts to lower egg yolk cholesterol to improve consumer perceptions of egg health quality. However, such n-3 enriched egg products must meet consumer demands with regard to sensory and functional characteristics before consumer health benefits could be realized.

Previously, we reported that consumers could detect a difference in scrambled eggs, but not hard cooked eggs, obtained from hens fed 3% menhaden oil (MO) as compared to those fed no-added-fat control rations (Van Elswyk et al., 1992). The ability to differentiate scrambled n-3 fatty acid-enriched eggs from control eggs was suggested to be related to differences in volatile compound concentrations (Van Elswyk et al., 1992).

Our objectives were: (1) to determine the sensory characteristics of eggs produced from hens fed graded levels of MO (0.0, 0.5, 1.5, and 3.0%) using a trained sensory panel; (2) to determine the volatile profile of cooked eggs from hens fed graded levels of MO; and (3) to correlate the evaluation of sensory characteristics with headspace volatile compounds.

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MATERIALS & METHODS

Hens, diets, and egg collection

We used 36-wk-old White Leghorn laying hens (136) divided into four dietary treatment groups (n = 30/treatment; 10/replication). Dietary treatments consisted of a typical layer ration containing graded levels of nondeodorized MO including 0, 0.5, 1.5, and 3.0%. Menhaden oil was stabilized with 500 ppm ethoxyquin by the supplier (Zapata Haynie, Reedville, VA 22539). Eggs were collected following 4 wk of feeding the n-3 fatty acid-enriched diets. Our previous research indicated that yolk n-3 fatty acid incorporation leveled off following 4 wk of feeding (Hargis et al., 1991). Eggs were collected during a 1-wk period (on 3 separate days) and held at 4°C until transport or analysis for fatty acid composition. Air transport of eggs was completed, unrefrigerated during a 6-hr flight. Upon arrival at Clemson University eggs were returned to refrigerated storage. Eggs for headspace analysis were subjected to an additional 8 hr unrefrigerated transport to North Carolina State University. In all cases attempts were made to insulate cooled eggs to reduce heat exposure (eggs were typically cool to the touch when unpackaged). Eggs were randomly selected (within replication) for fatty acid, taste panel, or headspace volatile analysis.

Fatty acid analysis

Yolks (n = 10) were pooled (n = 2) for each of 2 replicates (collection days) per treatment. Egg yolks were separated, weighed, pooled and blended. Four 1-g aliquots of yolk from each pool were extracted using chloroform/methanol (2:1, vol/vol) (Folch et al., 1957). In a similar manner, diet fatty acid content was also determined, using lipid extracts from 5-g samples of feed. For both egg yolk and feed, percentage fat was determined gravimetrically. Esters of fatty acids from all lipid extracts were then prepared using a 0.2g aliquot of each extract. Methanolic borontrifloride was the transesterifing agent (Van Elswyk et al., 1992). Fatty acid methyl esters (FAME) were quantitated by gas chromatography, using a column packed with GP 10% SP 2330 on 100/120 chromosorb in W AW (Supelco, Bellfonte, PA 16823). Samples were chromatographed at 150°C to 240°C with a 2°C/min programmed temperature gradient. Nitrogen was carrier gas at 30 mL/min. Fatty acid peaks were identified by comparison with retention times of FAME standards (reference mixture 6; 20:5n-3; 22:6n-3; Alltech, Deerfield, IL) and quantified by comparison with an internal standard area (C13:0; Alltech, Deerfield, IL) using Expert Chromatography System™ software (Version 4.0; Waters, Millford, MA 01757). Feed fatty acids were expressed as percent of lipid extracted from a 5-g feed sample, corrected to represent 1 g of feed (Table 1).

Sensory panel

Twelve panelists participated in three special training sessions, specific to scrambled eggs and fish oil, prior to evaluations. The Spectrum method of training to detect flavor intensity was utilized (Meilgaard et al., 1989). Panelists were informed that egg samples were from hens fed 3 treatment levels of non-deodorized MO. Panelists evaluated scrambled egg samples for ten aroma, nine taste, and six aftertaste characteristics using an intensity scale of 0 (not present), X (threshold), 1 (slight), 2 (moderate), to 3 (strong) (Table 2). Score card terms were previously utilized by Koehler and Jacobson (1966), and during the training sessions panelists were encouraged to suggest appropriate modifications. The threshold indication was later assigned a value of 0.5 for statistical anal-

Table 1—Fatty acid analysis of experimental diets

Fatty acid[a]	% Menhaden oil 0	.5	1.5	3.0
	(% Total fatty acids)			
16:0	16.7 ± 0.5	17.2 ± 0.5	19.5 ± 0.3	20.8 ± 0.9
16:1n-9	.4 ± 0.1	2.5 ± 0.3	3.2 ± 0.1	4.6 ± 0.6
18:0	6.8 ± 0.4	6.7 ± 0.5	6.7 ± 0.6	6.8 ± 0.5
18:1n-9	24.6 ± 0.7	24.8 ± 0.5	25.6 ± 0.9	25.8 ± 0.7
18:2n-6	49.2 ± 0.5	40.1 ± 0.1	37.5 ± 0.1	30.4 ± 3.9
18:3n-3	1.3 ± 0.6	1.5 ± 0.4	1.7 ± 0.8	2.4 ± 0.3
20:4n-6	1.0 ± 0.6	1.1 ± 0.5	1.2 ± 0.7	2.3 ± 1.7
20:5n-3	n.d.[b]	.5 ± 0.1	1.6 ± 0.1	4.5 ± 0.1
22:6n-3	n.d.	1.5 ± 0.1	2.7 ± 0.3	2.7 ± 0.2
Total n-6 fatty acids	50.2 ± 0.7	41.2 ± 1.2	38.6 ± 1.5	32.7 ± 2.2
Total n-3 fatty acids	1.3 ± 0.6	3.5 ± 1.1	6.0 ± 1.3	9.5 ± .5

[a] Mean ± SEM.
[b] Not detectable.

Table 2—Form for sensory evaluation of scrambled eggs from hens fed graded levels of dietary menhaden oil

AROMA[a]	TASTE[a]	AFTERTASTE[a]
Characteristics[b]	*Characteristics*	*Characteristics*
Sulfury	Sweet, mild	Fishy
Sweet, fragrant	Sulfury	Mouth-coating
Hydrolyzed protein	Earthy	Sweet
Acid	Brothy, meaty	Astringent, alkaline
Earthy	Acid	Acid
Fishy	Musty	Metallic
Chemical, medicinal	Fishy	Other
Musty	Chemical, medicinal	
Ammonia	Stale, rancid	
Noseburn	Other	
Other		

OVERALL QUALITY

What is your estimate of *overall* quality of this sample of egg, based on its *flavor*?

Inedible					Edible					Very good
⊥	⊥	⊥	⊥	⊥	⊥	⊥	⊥	⊥	⊥	⊥
0	1	2	3	4	5	6	7	8	9	10

[a] Intensity scale of 0 - not present, .5 - threshold, 1 - slight, 2 - moderate, 3 - strong.
[b] Adapted from Koehler and Jacobson, (1966).

Table 3—Egg yolk fatty acid content in response to graded levels of dietary menhaden oil

Fatty Acid	% Dietary menhaden oil 0	0.5	1.5	3.0
	(mg/g yolk)			
16:0	65.7 ± 4[a]	74.6 ± 15[a]	74.5 ± 3[a]	73.5 ± 4[a]
16:1n-9	9.7 ± 0.8[a]	11.0 ± 2[a]	11.3 ± 0.4[a]	11.2 ± 0.8[a]
18:0	21.3 ± 2[a]	24.1 ± 5.3[a]	24.1 ± 1[a]	23.5 ± 1.3[a]
18:1n-9	115 ± 8[a]	131 ± 5[a]	131 ± 6[a]	125 ± 4[a]
18:2n-6	29.5 ± 1[a]	37.9 ± 5[a]	34.7 ± 2[a]	30.8 ± 3[a]
18:3n-3	.40 ± 0.1[b]	.80 ± 0.04[a]	1.1 ± 0.05[a]	1.0 ± 0.2[a]
20:4n-6	4.8 ± 0.5[b]	5.1 ± 0.6[b]	2.9 ± 0.1[a]	2.2 ± 0.2[a]
20:5n-3	< 1[c]	.31 ± 0.2[c]	.72 ± 0.1[b]	1.2 ± 0.1[a]
22:6n-3	1.71 ± 0.2[c]	6.03 ± 1.4[b]	9.57 ± 0.4[b]	10.4 ± 1.3[a]
Total n-3	2.12 ± 0.2[c]	7.19 ± 1.5[b]	11.4 ± 0.5[a]	12.6 ± 1.6[a]

[abc] Means ± SEM significantly differ within row If superscripts differ.

ysis. The standard for high quality was determined using eggs from hens fed a typical layer ration. A total of three replications were completed on three consecutive days with two evaluations per panel and 1 hr between evaluations. Nonflavored, carbonated water was available to panelists at room temperature (≈25°C).

Sample preparation

Three randomly selected eggs from within each replication of dietary treatment were broken into a coded bowl and whipped with a wire whisk. Eggs were prepared in pre-heated Silverstone Premium Non-stick™ electric skillets (Sunbeam-Oster Inc., Providence, RI, 02903) with set at 122°C. Samples were cooked for 4 min then served in coded paper cups containing ≈ 25g scrambled egg. All panelists were presented samples immediately following cooking. Treatments were served simultaneously in random order.

Headspace gas chromatography

Whole eggs (n = 10/trt) were pooled (n = 3/trt, 1/rep) and blended for ≈ 20 sec in a blender flask to produce a consistency resulting in a 10 sec flow from a Number 3 Zahn viscometer cup (General Electric Co., West Lynn, MA 01903). A glass Erlenmeyer flask (150 mL) was held in the vortex to minimize foaming and the incorporation of air into the blended egg. Duplicate 2-g samples of blended egg were taken from each pool and pipetted into a 5 mL headspace vial and sealed with a teflon-lined silica septum (Perkin-Elmer, Norwalk, CT 06859). Individual sample vials were heated at 100°C for 30 min followed by a 4 min pressurization and 1 min injection sequence using a Perkin-Elmer HS-6 headspace sampler interfaced with a Varian Model 3700 dual-flame gas chromatograph (Varian, Sunnyvale, CA 94089). Volatiles were injected onto a 30m × .32mm i.d. bonded phase fused silica capillary column DB-5 (J & W Scientific, Folsom, CA 95630) with a 1-µm film thickness. The column inlet pressure was 138kPa, helium was the carrier gas with a 7.44:1 injection split ratio and 6.2 mL/min flow rate. The cryogenically cooled column oven temperature was programmed from −30°C to 220°C at 4°C/min with 1 min hold time. The injector was 230°C and detector 240°C. Gas chromatographic peaks were integrated using a Waters 820 chromatography data station (Waters, Millford. MA 01757).

Volatiles were tentatively identified on the basis of retention times of reference standards (Polyscience Corp., Niles, IL 38452). Confirmation of volatile identities was accomplished on a Hewlett-Packard Model 5987 GC-MS (Hewlett Packard, Avondale, PA 19311). Volatiles were introduced into the GC-MS with a OSC-I purge and trap unit (Tekmar, Cincinnati, OH 18432). The ionization energy was 70 eV, and a scan range of 30 -300 m/z was used. Spectra were identified primarily by comparing with published spectra.

Statistical analysis

Fatty acid content was analyzed using the general linear model (GLM) of Statistical Analysis Systems (SAS Institute, Inc., 1984) with treatment × replication as the error term. Significant differences among dietary treatment means were determined by the Duncan procedure (SAS Institute, Inc., 1984). The taste panel scores and GC headspace volatile peak areas (three replications) were analyzed using the GLM with the level of dietary MO (treatment) as the main effect. The treatment × replication interaction was used as the error term and significant differences among means was determined by the least square means procedure (Helwig and Council, 1982). Correlations between headspace volatiles and sensory notes were determined using Pearson Correlation Coefficients (SAS Institute, Inc., 1984).

RESULTS & DISCUSSION

Fatty acid profile

All levels of dietary MO resulted in significant alterations in total raw egg yolk fatty acid composition (Table 3). Diet did not influence total saturated or monounsaturated fatty acids (Table 3). Alterations due to dietary MO levels were noted among polyunsaturated fatty acids of the n-6 and n-3 series. However, all MO levels did not result in the same magnitude of change in the raw egg yolk n-6/n-3 fatty acid composition.

Dietary administration of 1.5 and 3.0% MO resulted in 40% and 54% decrease in arachidonic acid (20:4n-6), respectively. These results were similar to previously reported data which indicated a 70% average reduction in total n-6 fatty acids in response to 3% dietary MO (Hargis et al., 1991). The administration of 0.5% dietary MO did not alter arachidonic acid content of the yolk.

Total n-3 fatty acids increased regardless of dietary MO levels although level of incorporation was greater in response to 1.5% and 3.0% dietary MO compared to the 0.5% level. Linolenic acid (18:3n-3) was increased twofold in response to 0.5%, and 2.5-fold in response to 1.5% and 3.0%. Docosahexaenoic acid (22:6n-3) was increased threefold in 0.5% eggs, and sixfold in

the 1.5% and 3.0% dietary levels. Eicosapentaenoic acid (20:5n-3) was not detectable in eggs from hens fed the typical layer ration. However, levels of 5, 14, and 25 mg/egg were found in egg yolks from hens fed MO rations containing 0.5%, 1.5%, and 3.0%, respectively. These findings were also consistent with our previous report which indicated a 6-fold increase in DHA and incorporation of 15-28 mg of EPA/yolk utilizing 3.0% dietary MO (Hargis et al., 1991).

The level of total n-3 fatty acids incorporated into the egg yolks from hens fed 1.5 and 3.0% dietary MO did not differ significantly (Table 3). The level of dietary n-3 fatty acids needed to positively affect risks of coronary heart disease is not clear (Drevon, 1992; Kromhout, 1992; Barlow and Pike, 1991). The amount of dietary n-3 consumed from one n-3 enriched shell egg (1.5% and 3.0% dietary treatments), would provide about the amount attainable from a 100 g serving of lean white fish such as cod (USDA, 1986).

Sensory panel

Five of the sensory characteristics evaluated were affected by level of dietary MO (Table 4). Therefore results reported of the effects of dietary MO on egg sensory quality will be limited to fishy aroma, fishy taste, fishy aftertaste, sweet taste, and medicinal taste.

Sweet taste was lower in 0.5% eggs as compared to other dietary treatments (Table 4). A sweet flavor and aroma has been identified as a contributor to flavor of fresh scrambled eggs (Warren and Ball, 1991). This sweet flavor has been strongly associated with yolk although the interaction of yolk and white is important for identifying acceptable egg flavors (Warren and Ball, 1991).

Fishy taste, fishy aroma, and fishy aftertaste increased, generally in a dose-response fashion, as level of dietary fish oil increased (Table 4). Fishy taste was greater in eggs from hens fed 1.5% and 3.0% MO as compared to 0.5% and control eggs. Fishy aftertaste was greater in 3.0% eggs as compared to controls, but was not different from the intermediate levels of MO (Table 4). Fishy aroma was increased in eggs from hens fed 3.0% MO as compared to 0.5% and control eggs. However, fishy aroma scores were not different between eggs from hens fed 1.5% or 3.0% MO (Table 4). A decrease in overall flavor scores corresponded with increases in fishy characteristics. Overall flavor inversely correlated with fishy aroma (-0.88, $P < 0.001$), fishy taste (-0.91, $P < 0.0001$), and fishy aftertaste (-0.91, $P < 0.0001$). However, only the 3.0% treatment was significantly lower in overall flavor quality (data not shown). This finding was expected as the perception of fishiness may often be considered undesirable, even in seafood products (Lindsay, 1990).

The effects of MO on egg flavor were consistent with previous findings utilizing a consumer sensory panel and eggs from hens fed 1.0, 2.25, and 4.75% MO (Holdas and May, 1966). They detected off-flavors in soft-cooked eggs from hens fed the highest level of MO but not with lower levels of dietary MO. They concluded that 1.0 and 2.25% dietary MO did not adversely affect egg flavor. While fishy characteristics in our study inversely correlated with overall quality, the increase in fishy aroma, taste, and aftertaste did not fully account for reduced flavor acceptability between the 1.5% and 3.0% MO treatments.

The chemical or medicinal flavor note was greater in eggs from hens fed 3.0% MO as compared to those from control and 0.5% MO eggs. Medicinal taste in eggs from hens fed 1.5% was not greater than 0.5% and control. Despite the wide variation in scores between eggs from hens fed MO at 1.5% (.09) and 3.0% (.23), this note was not different between the treatments. A chemical or medicinal taste in eggs was first identified by Koehler and Jacobson (1966) who, investigated relationships between flavor characteristics in whole egg, whole yolk, and various yolk fractions prepared by ultracentrifugation. Several yolk fractions were identified as having a chemical or medicinal taste, which was not detectable in whole egg or unfractionated yolk.

Table 4—Effect of graded levels of dietary menhaden oil on sensory characteristics of scrambled eggs

		Sensory characteristics			
Diet	Fishy aroma[d]	Sweet taste[d]	Fishy taste[d]	Medicinal taste[d]	Fishy aftertaste[d]
0.0%	0.4[b]	2.4[a]	0.3[c]	0.06[b]	0.3[b]
0.5%	0.4[b]	1.6[b]	0.6[bc]	0.01[b]	0.8[ab]
1.5%	0.8[ab]	2.1[a]	0.9[ab]	0.09[ab]	0.7[ab]
3.0%	1.0[a]	2.1[a]	1.2[a]	0.23[a]	1.1[a]

[a,b,c] Means with different superscripts within columns are significantly different ($P < 0.05$).

[d] A 0 to 3 intensity scale was used with 0 = not present, .5 = threshold, 1 = slight, 2 = moderate, and 3 = strong.

Headspace volatiles

The concentration of 23 of the 42 volatiles separated from heated whole egg were affected by level of MO in the diet (Table 5). No volatile compounds, unique to eggs from hens fed MO were identified indicating that changes in headspace volatile profiles due to MO were quantitative. 3-Methybutanal was not affected by diet, however, this volatile was identified as the most abundant in the headspace profile, confirming earlier results with cooked egg (MacLeod and Cave, 1975). With exception of chloroform, volatile compounds of low molecular weight increased (or remained unchanged) as level of dietary MO increased (Table 5). Specifically, unknowns #1 and #2, (retention times 0.58 and 5.4) were lowest in control eggs and increased in a stepwise manner as MO level increased (Table 5). Acetone increased in MO-enriched eggs and was highest in eggs from hens fed 1.5 and 3.0% MO. Unknowns #3 and #4, retention times 7.3 and 7.9, were greater in MO eggs as compared to controls. Unknown #3 was highest in eggs from hens fed 1.5 and 3.0% MO. Hexane was increased in response to dietary MO regardless of level (Table 5). Hexane has been previously identified in meat samples from turkeys fed 2.0% tuna oil (Crawford and Kretch, 1976).

While low molecular weight headspace volatiles tended to increase with increased dietary MO, higher molecular weight volatiles tended to decrease. Hexanal was highest in control eggs and decreased with added MO, with lowest concentrations present in eggs from hens fed 1.5 and 3.0% MO. Hexanal has been reported in scrambled egg samples and had been identified as a product of thermal lipid oxidation (Matiella and Hsieh, 1991). Boyd et al. (1992) suggested that hexanal may not be an appropriate indicator of n-3 fatty acid degradation. They noted that hexanal correlated more directly with linoleic acid degradation. That hypothesis may explain the pattern of hexanal changes noted between control and n-3 fatty acid-enriched shell eggs in our study.

Decreased hexanal concentration of n-3 fatty acid enriched shell eggs, while probably not related to n-3 fatty acid degradation, may have contributed to the decreased intensity of sweet flavor characteristic of eggs from hens fed .5% MO. Hexanal has previously been identified by stepwise discriminant analysis as positively correlated with sweet and dairy aroma and flavor notes of fresh scrambled eggs (Warren, 1994).

Autoxidation products of pure DHA (22:6n-3) have been previously identified by Noble and Nawar (1975). They identified 2-pentyl furan as a major degradation product of DHA. In our study, 2-pentyl furan was reduced as the level of dietary MO fed to hens increased, being lowest in eggs from hens fed 3.0% dietary MO. Boyd and coworkers (1992) suggested that propanal and propenal were appropriate indicators of n-3 fatty acid oxidation. These compounds were not identified in our study. Boyd and co-workers reported, however, that those compounds were most prevalent following 65 min heating. Samples were heated only 30 min in our study. Additionally, they were work-

Table 5—Least square means for headspace volatile compound peak areas from heated liquid whole egg from hens fed graced levels of menhaden oil

			% Dietary menhaden oil				
Compound[e]	ID[f]	RT[g]	0.0%	0.5%	1.5%	3.0%	SEM
Unknown 1	—	0.58	9006^{c}	11463^{b}	13422^{a}	14340^{a}	645
Acetylaldehyde	MS,RT	1.92	42289	45406	46485	49345	
Unknown 2	—	5.4	285^{c}	407^{b}	459^{ab}	526^{a}	33
Acetone	MS	5.7	32620^{b}	31427^{b}	42033^{ab}	45484^{a}	2511
Diethyl ether	MS	6.4	987	1005	900	833	
Dimethyl sulfide	MS	7.0	1599	1476	1509	1476	
Unknown 3	—	7.3	146^{c}	356^{b}	516^{a}	520^{a}	50
Unknown 4	—	7.9	282^{b}	583^{a}	768^{a}	652^{a}	65
2-Methyl propanal	MS,RT	9.1	18714	19071	19766	20676	
Thirane	MS,RT	9.7	136	121	139	184	
Butanal	MS,RT	11.3	7419	7185	7375	7648	
Hexane	MS,RT	11.6	157^{b}	174^{ab}	237^{ab}	294^{a}	21
Chloroform	MS,RT	11.9	173^{a}	90^{bc}	109^{b}	41^{c}	18
Ethyl acetate	MS,RT	12.4	78	122	194	231	
Methylcyclo pentane	MS,RT	13.3	1332^{b}	5175^{a}	2101^{b}	1590^{b}	860
3-Methyl butanal	MS,RT	14.6	48575	47413	49803	52980	
2-Methyl butanal	MS,RT	15.1	17194	17888	18562	19282	
Unknown 5	—	16.4	950^{d}	2503^{c}	3357^{b}	3963^{a}	354
Pentanal	MS,RT	17.0	16551^{a}	12235^{b}	9472^{c}	8301^{c}	1011
Heptane	MS,RT	17.3	448^{c}	1406^{b}	1731^{ab}	2286^{a}	243
Dimethyl disulfide	MS	19.4	1138	1079	1102	1132	
Unknown 6	—	20.2	139^{c}	239^{b}	322^{a}	388^{a}	32
Toluene	MS,RT	20.7	67	125	118	132	
Unknown 7	—	21.1	318^{a}	1421^{b}	931^{bc}	864^{c}	341
Hexanal	MS,RT	22.6	40445^{a}	28562^{b}	21862^{c}	19547^{c}	2553
Unknown 8	—	24.1	1388^{a}	872^{b}	586^{c}	461^{d}	110
Unknown 9	—	24.2	1526^{a}	1080^{b}	855^{c}	783^{c}	93
Unknown 10	—	27.2	750^{a}	504^{b}	566^{b}	307^{c}	60
Unknown 11	—	27.7	65^{c}	406^{b}	512^{ab}	718^{a}	87
Heptanal	MS,RT	27.8	3465	2975	3827		3133
Benzaldehyde	MS	30.4	277	227	153	174	
Trimethyl benzene	MS	30.5	2006	2240	2166	2359	
Octenol	MS	31.5	661^{a}	344^{b}	190^{c}	216^{bc}	68
2-Pentyl furan	MS,RT	32.1	2744^{a}	1985^{ab}	2039^{ab}	1440^{b}	181
Octanal	MS,RT	32.6	2700	2613	3036	2770	
Unknown 12	—	33.9	347^{a}	278^{a}	162^{b}	164^{b}	26
Unknown 13	—	35.0	239^{a}	276^{a}	132^{b}	127^{b}	25
Heptonate	MS	36.9	75	54	66	84	
Nonanal	MS,RT	37.1	3302	3556	3541	3468	
t-2-Nonenol	MS	40.2	525	594	562	513	
Unknown 14	—	40.8	198^{a}	212^{a}	50^{b}	46^{b}	30
2,4,Decadienal	MS,RT	41.3	251	249	241	235	

[abcd] Means with similar superscripts are not different ($P > 0.05$).
[e] In order from lowest to highest molecular weight.
[f] Identification method; MS = mass spectrometry; RT = retention times.
[g] Represents retention time in minutes.

ing with purified fatty acids, and a mixed system was evaluated in our study. A similar explanation may apply concerning the conflicting results of our study with Noble and Nawar (1975). Unknowns identified by retention times of 24.1 (#8), 24.2 (#9), and 27.2 (#10) decreased in relation to increasing dietary MO.

More important than the effect of diet on individual compounds associated with lipid oxidation may be the quantitative shifts in volatile headspace profiles noted in response to dietary MO. These shifts may be reflective of the increased degree of unsaturation in n-3 fatty acid-enriched eggs. The rate of oxidative degradation increases with degree of unsaturation (Pearson et al., 1977). This may account for the increase in lower molecular weight (MW) and decrease in higher MW volatile compounds from cooked eggs from hens fed the 1.5 and 3.0% levels of MO (Table 5).

Individual compounds contributing to formation of fishy flavor have been difficult to identify. Previous investigators concluded that the flavor perceived as "fishy" resulted almost entirely from a complex interaction of compounds (Badings, 1973; Crawford and Kretch, 1976). They also concluded that many compounds which have been associated with fishy flavor have low odor thresholds, indicating very minor changes in concentrations may result in detectable off-flavors. For example, the combination of n-hexanal, n-heptanal, hex-2-enal, and oct-1-en-3-one was believed responsible for the fishy flavor of oxidized butterfat (Pont et al., 1960; Forss et al., 1960; Stark and Forss, 1962) and salmon oil (Yu et al., 1961). The combined influence of 2,4-heptadienals, 2,4-decadienals, and the 2,4,7-decatrienals have been suggested as indicative of oxidizing fish oil (Josephson, 1991). In our study, only 2,4-decadienal was identified and it was not influenced by the presence of yolk n-3 fatty acids.

Additionally, the position of the double bond in fatty acids may influence the flavor profile of compounds produced during oxidation. This can be illustrated in the difference in aroma thresholds of 2 vinyl ketones; octa-1-cis-5-dien-3-one and oct-1-en-3-one. The 5-diene, an oxidation product of n-3 pentaenoic acid, was detected at 100 times lower concentrations in both oil and water than oct-1-en-3-one, (an oxidation product of n-6 pentaenoic acid, Swoboda and Peers, 1977). The low thresholds of compounds from n-3 fatty acid oxidation may have influenced the sensory panel detection of fishy flavor and perception of flavor quality in eggs from hens fed 3% MO.

Early investigators also theorized that interactions of nitrogenous and lipophilic compounds led to formation of fishy flavors. Davies and Gill (1936) theorized that fish odors from fish oils, linseed oil, and butterfat were derived via a two-step process. Tertiary nitrogenous compounds reacted with unsaturated fatty acids in the oil and the fishy odor developed upon oxidation. In those studies, trimethylamine, trimethylamine oxide, or choline were probably the nitrogenous source (Davies and Gill,

Table 6—Correlation between sensory panel characteristics and headspace volatile concentrations

Volatile compound[a]	Sensory panel characteristic: Fishy aroma	Sweet taste	Fishy taste	Fishy aftertaste
0.58	0.68**	0.68**	0.81**	0.77**
5.4	—	−0.64*	0.65*	0.57*
acetone	0.56*	—	—	—
7.27	0.68**	−0.61*	0.73**	0.66**
hexane	0.59*	−0.61*	0.60*	0.59*
16.4	0.62*	−0.69*	0.79**	0.71**
pentanal	−0.73**	0.69*	−0.76**	−0.66**
heptane	—	−0.64*	0.66*	0.63*
21.0	0.64*	−0.66*	0.82**	0.80**
21.1	—	—	−0.75**	−0.65*
hexanal	−0.71**	0.63*	−0.84**	−0.74**
24.1	−0.74**	0.69**	−0.84**	−0.72**
24.2	−0.72**	0.66*	−0.83**	−0.72**
27.2	−0.68**	0.81	−0.58*	—
27.7	0.59*	−0.75**	0.61*	0.57*
octenal	−0.70**	0.70**	−0.62*	—
2-pentyl furan	−0.62*	0.67**	−0.58*	—
33.9	−0.73**	0.60*	−0.72**	−0.64*
35.0	−0.79**	0.60*	—	—
40.75	−0.66**	—	—	—

[a] Identification of volatile compound by retention time.
— Nonsignificant correlation coefficient * Significance at $P \leq 0.05$; ** Significance at $P \leq .01$.

1936). Preliminary GC-MS data from our lab indicates that unknown 11 (retention time 27.7) may be trimethylamine. This compound increased in a step-wise manner as level of dietary MO increased (Table 5). From the data we could not definitively identify specific compounds and interactions responsible for observed off-flavors. However, it seems likely that some or all of the above oxidation products were contributory to increased fishy notes and associated decreases in overall quality of eggs from hens fed the highest (3.0%) levels of MO.

Correlation of sensory panel characteristics and volatile compounds

For the most part, volatiles which positively correlated with fishy notes also negatively correlated with sweet taste (Table 6). Four volatile compounds (retention times 0.58, 7.27, 16.4, and 21.0 min) negatively correlated with sweet taste, typical of fresh eggs (Table 6). Hexanal negatively correlated with fishy notes suggesting a weak association between hexanal and n-3 fatty acid degradation as has been suggested (Boyd et al., 1992). The volatile concentration data (Table 5) revealed that only 1 volatile (unknown #5, retention time 16.4) was higher in concentration in the 3.0% compared to the 1.5% treatment eggs. This volatile positively correlated with fishy characteristics and negatively correlated with sweet taste (Table 6). It is unlikely that this single compound was solely responsible for increased sensory panel detection of off-flavors in eggs from hens fed 3.0% MO.

CONCLUSIONS

UTILIZATION OF 1.5 DIETARY MO or more resulted in incorporation of n-3 fatty acids into egg yolk. Due to the pattern of changes in the volatile compound profile associated with n-3 fatty acid enrichment, oxidation of these fatty acids is probably contributory, to off-flavor development. Further elucidation of changes in volatile compounds associated with n-3 enrichment of shell eggs, and their effects on product quality may be critical for maintaining overall flavor quality of eggs and consumer acceptability of such products.

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Instrumental Method for Characterizing Protein Foams

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ABSTRACT

A whipping machine for cream analysis (Cream Tester CTII) was used to foam egg white, milk proteins and soy protein isolate. Foam formation and final rigidity were characterized by the current input to the beating motor. In addition, specific volume, rigidity (compressive strength by Instron) and stability (drainage) of the foams were determined by methods that minimized foam damage during handling. Current input during whipping distinguished fresh vs pasteurized egg white and positively correlated with compressive strength of final egg white foams. Such correlation was not found for milk protein and soy protein isolate foams of lower strength and stability.

Key Words: protein, foam, egg white, milk, SPI

INTRODUCTION

FOAM FORMATION is an important functional property of egg white and of some milk proteins. To produce and characterize protein foams, various methods have been reported. Foams have been formed by a blender- (Britten and Lavoie, 1992) or mixer-type whipping apparatus (Cotterill et al., 1992; Gassmann et al., 1987; Phillips et al., 1987) or by sparging gas through the protein solution (Bastiaens et al., 1990). Foaming properties have been measured by foaming capacity and foam stability (German et al., 1985; Mulvihill and Fox, 1987). A standardized procedure for measuring foaming properties of proteins was reported by Phillips et al. (1990). No commonly used method follows development of foam rigidity during whipping. To find the maximum foam rigidity as related to whipping time, a series of protein samples must be foamed for different times. In addition, depending on the method, sample preparation for overrun, rigidity and drainage measurements can result in partial destruction of the foam.

In the dairy industry, cream quality is often evaluated using the MOHR-type whipping system (Hinrichs et al., 1992; Hungarian Standard, 1980; Lorenzen et al., 1993; Schweizerisches Lebensmittelbuch, 1970). This apparatus whips cream at a constant temperature until maximum firmness is reached and then automatically stops to avoid overbeating, characterized by decrease in foam firmness. Foam firmness is measured by the current input to the motor (mA) during beating. The overrun is directly determined with minimal damage by the height of the whipped cream in the whipping beaker. However, for drainage measurements the foam is filled into a cube where a partial breakdown of foam structure due to mechanical stress may occur.

Our objective was to test the suitability of the MOHR-type whipping system to foam protein solutions using egg white, milk proteins and soy protein isolate. Foams were characterized by specific volume, rigidity and stability, using methods that were modified to minimize foam damage.

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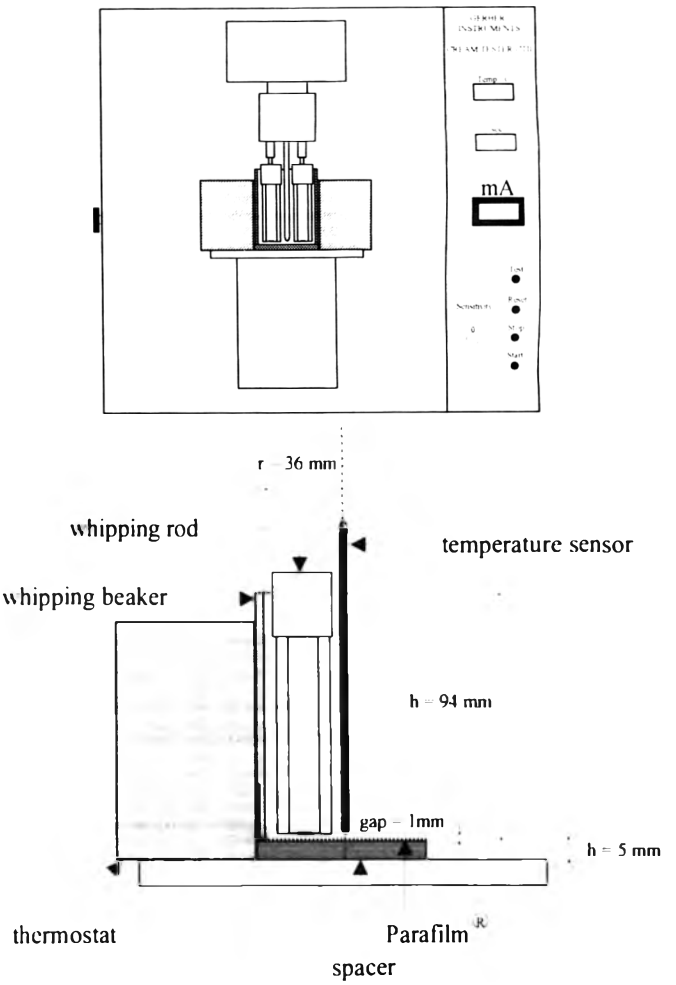


Fig. 1—Cream Tester CTII and detail of whipping system.

MATERIALS & METHODS

Whipping apparatus

The whipping machine (Cream Tester model CTII, Gerber Instruments, K. Schneider & Co. AG, Effretikon, Switzerland) was designed to measure and record the current input to the motor as a function of whipping time. Current is proportional to energy required to maintain rotation speed (300 rpm). It is 143 mA for a run without product. Whipping solutions will cause the current to increase with increasing rigidity of developing foam. In the automatic switch-off mode, the machine stops when a slow (sensitivity 9) or a fast (sensitivity 1) decrease in current input occurs. A decrease in current input is due to reduction of foam rigidity and therefore corresponds to a breakdown of foam structure by overbeating. Since this decrease in current was not observed with two exceptions (micellar casein and skim milk), the machine was stopped manually (sensitivity 0) once a plateau (stable current input) was reached. The final current was used as a measure of foam rigidity. For safety reasons the Cream Tester was set to stop after 3.5 min. That value is suitable for analysis of cream but for protein solutions the time was extended to 25 min by manipulation of the whipping program. A temperature sensor may be incorporated between the two whipping rods (Fig. 1) to measure and record sample temperature during beating. Foams that had to be analyzed further were produced without the temperature

sensor. The original sample beaker was replaced with a tube of identical dimensions. The tube was closed at the bottom with Parafilm® (American National Can, Greenwich, CT). A spacer was designed to assure a gap of 1 mm between the beaker bottom and the whipping rods. A water jacket at 25°C surrounded the beaker. Whipping was performed with 40 g of protein solution. To determine current input baselines, each product was also whipped after addition of octanol to prevent foam formation.

Products

Commercial samples of fresh and pasteurized egg white (Lüchinger & Schmid, Zürich, Switzerland) were diluted to (10%) total solids (TS) prior to foaming. Both samples were pH 9.2. A dilution series from 12.2 %TS to 4.9 %TS was prepared using another batch of pasteurized egg white. Powders of micellar casein (skim milk retentate micro- and diafiltered on a Techsep/Rhone Poulenc M14 membrane, Miribel, France), skim milk (medium heat; Berneralpen Milchgesellschaft, Konolfingen, Switzerland), defatted total milk protein (Prodiet 752; Ingredia, Arras, France), κ-caseinate (Emmi, Dagmarsellen, Switzerland), whey protein isolate (Bipro; Le Sueur Isolate, Le Sueur, MN) and soy protein isolate (Edipro A; Protein Technologies, St. Louis, MO) were dissolved in distilled water. The solutions were stirred at 50°C for 1 hr (except whey and soy protein isolate stirred at room temperature). After cooling, the pH was adjusted to 7.0 where necessary with 1N HCl or 1N NaOH. Final dilution gave 10 %TS (in addition, 5 %TS for skim milk powder and κ-caseinate). Products were stored overnight at 5°C before analysis. In addition, pasteurized egg white was mixed with whey protein isolate solution (both at 10 %TS) in a weight ratio of 80/20, 50/50 and 20/80.

With exception of total milk protein and soy protein isolate, all products were completely dispersed. No protein sediment occurred after centrifugation at 40 × *g* for 10 min (Heraeus AG, Labofuge 6000, Zürich, Switzerland). The protein concentration in the supernatant was about 80% of the original concentration for total milk protein and 25% for soy protein isolate.

Measurement of foam characteristics

For egg white foams the Parafilm® was removed after whipping and once detached from the wall with a small spatula, the foam was gently pushed out of the whipping beaker with a plunger (Fig. 2). A 5 mm slice was cut and rejected. The second slice of 3 cm was used to determine specific volume, firmness and drainage rate for each sample. As a measure of overrun, the specific volume (mL/g) of the foams was determined from the weight of the 3 cm foam slice (height given by a reference tube, see Fig. 2). Overrun could be calculated from the relative increase in specific volume due to whipping (% overrun = 100 × [specific volume of the foam-specific volume of the unwhipped solution]/ specific volume of the unwhipped solution). Foam firmness was determined with an Instron Universal Testing Machine Model 1122 (Instron Corp., Brucks, UK) equipped with a round plate compression probe (ø = 2.5 cm) and a 2 kg load cell. Cross head speed was 10 mm/min. Force in N was determined for a 15 mm compression. The cutting procedure and Instron measurement were performed within 2 min to assure minimal change in foam structure. Drainage was evaluated from the weight of drained liquid from the foam placed on a sieve (No. 20/27; Emil Hitz AG, Bassersdorf, Switzerland) up to 120 min at room temperature (≈23°C). Drainage time started immediately after whipping. Drainage was expressed as % of initial foam mass drained.

For other protein solutions and pasteurized egg white mixed with whey protein isolate solution, the evaluation was done without removing the foam from the beaker. The specific volume of foam was determined from the total foam volume (height of foam in beaker). Compression tests (Instron) were done on the foam in the beaker and drainage rate was evaluated by removing the Parafilm® over the beaker and placing the filled beaker on the sieve. Specific volume was not determined and drainage measurements were not made on the dilution series with pasteurized egg white.

Statistical analysis

Foam formation and analysis were performed in triplicate. Average values, the 95% confidence interval and coefficient of variation were determined. Statistical differences in results of current input and compressive force were determined using an analysis of variance in conjunction with a Newmann-Keuls test.

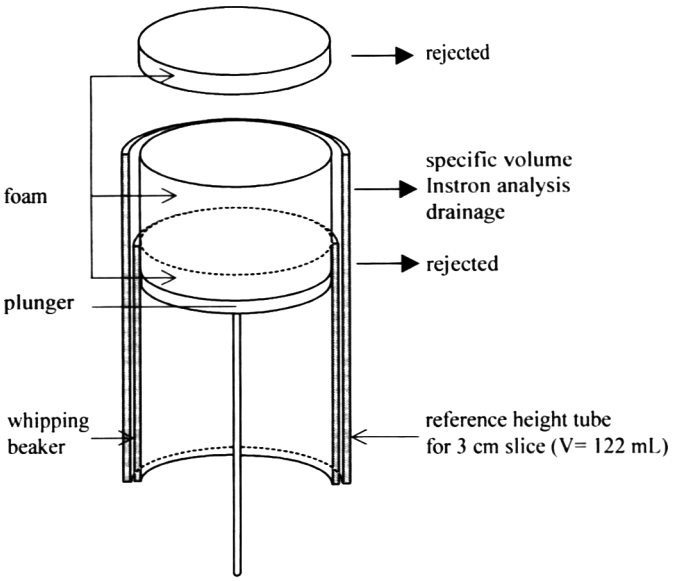


Fig. 2—Scheme for handling egg white foams.

RESULTS & DISCUSSION

INDIVIDUAL BEATING TIME, maximum current input, specific volume and compressive force were compared for all samples (Table 1). Typical records of current input during whipping (Fig. 3) for egg white, solutions of most milk proteins and soy protein isolate were compared. Not included are κ-caseinate at 10% TS due to the high final mA value and skim milk at 5% TS that demonstrated a curve shape very similar to the 10% TS solution but at a slightly lower current input level. The baseline current input for the whipping process in presence of octanol varied in height depending on viscosity of the solution (220 mA for κ-caseinate at 10% TS, 156 mA for fresh egg white, 150 mA for pasteurized egg white, 145 to 150 mA for all the other products, 145 mA for pure water). Since the values were very similar for most products, the baseline for water was used as a reference. The curves indicate the development of the foams. A continuous increase in current input occurred until a nearly stable value was reached. In most cases the current remained unchanged even over very long beating times. Thus, no breakdown of the foam occurred from overbeating. However, a maximum current input occurred at about 75 sec beating time for micellar casein and at about 100 sec for skim milk. The shape of the current input/time plot and the final maximum current were quite different and characteristic of individual protein solutions. This information may be applicable for further interpretation of foaming behavior. Thus, fresh and pasteurized egg white could be easily distinguished. Fresh egg white showed a higher final current after an initial lag phase where a slight decrease in current was found. This may reflect a homogenization of the thick and thin parts of egg white before foaming began. Total milk protein and soy protein isolate foams may be distinguishable by the shape of the current input/time plots and not by their maximum current. All curve shapes of egg white/whey protein isolate mixes (Fig. 4) were distinctly different from the curve shapes of individual protein solutions. The 50/50 (w/w) and 80/20 (w/w) mixes did not differ in their maximum current, but both foams were distinctly more rigid than the pasteurized egg white foam. Therefore the firmness of egg white foams may be enhanced by replacing up to 50% by whey protein isolate.

With κ-caseinate at 10% TS, a further increase in current was observed after the first intermediate plateau. Since the product became too stiff to whip to a final maximum, beating was stopped when the first plateau was reached. When foaming micellar casein, a part of the solution always stayed unwhipped at the bottom of the beaker. Therefore, multiple analysis of those products resulted in realtively large confidence intervals for cur-

Table 1—Foaming characteristics of protein solutions (10% TS)

Protein	Protein in solution (%)[a]	Whipping time (s)	Current input Mean (mA) ± conf. interval[d]	CV[e] (%)		Specific volume Mean (mL/g) ± conf. interval[d]	CV[e] (%)	Compressive force mean (N) ± conf. interval[d]	CV (%)	
Micellar casein	8.40	75	204 ± 9	1.77	A[c]	3.15 ± 0.45	5.59	0.048 ± 0.015	11.95	A[f]
Skim milk	3.47	100	189 ± 3	0.61	B	3.59 ± 0.15	1.63	0.052 ± 0.007	5.59	A
Skim milk (5% TS sol.)	1.74	100	179 ± 3	0.46	C	4.04 ± 0.15	1.19	—c		
Total milk protein	7.55	200	259 ± 7	1.02	D	4.54 ± 0.15	1.31	0.067 ± 0.007	4.33	A
κ-caseinate	8.41	150[b]	545 ± 10	0.74	E	2.58 ± 0.15	2.28	0.048 ± 0.007	5.97	A
κ-caseinate (5% TS sol.)	8.41	360	290 ± 4	0.53	F	5.57 ± 0.28	1.92	—c		
Whey protein isolate	9.03	660	244 ± 3	0.58	G	5.34 ± 0.13	1.35	0.140 ± 0.025	5.24	B
Soy protein isolate	8.69	1320	259 ± 5	0.80	D	3.93 ± 0.15	1.49	—c		
Fresh egg white	8.43	300	335 ± 6	0.69	H	5.81 ± 0.23	1.60	0.620 ± 0.015	0.93	C
Pasteurized egg white	8.37	300	270 ± 1	0.21	I	6.20 ± 0.38	2.38	0.500 ± 0.053	4.19	D
Pasteurized egg white/ Whey protein isolate										
20/80 w/w	8.90	360	265 ± 5	0.79	J	5.66 ± 0.30	2.07	0.190 ± 0.025	5.26	E
50/50 w/w	8.70	360	280 ± 3	0.36	K	6.07 ± 0.15	0.97	0.360 ± 0.053	5.73	F
80/20 w/w	8.50	360	282 ± 8	1.14	K	6.10 ± 0.45	2.89	0.320 ± 0.053	6.57	G

[a] N*6.38 for milk proteins; N* 6.25 for egg white and soy protein isolate.
[b] Intermediate plateau.
[c] Not measured due to the low stability;
[d] Conf. interval: 95% confidence interval;
[e] CV (%): coefficient of variation.
[f] A-K: means in the same column sharing the same letter are not statistically different (alpha = 0.05)

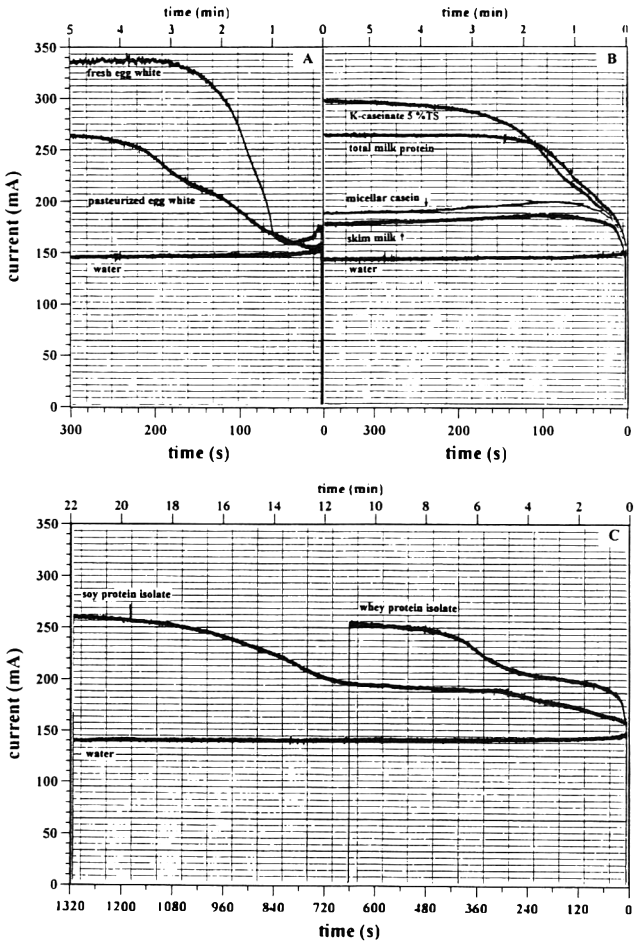


Fig. 3—Current input as related to whipping time for egg white, milk proteins and whey and soy protein isolate solutions.

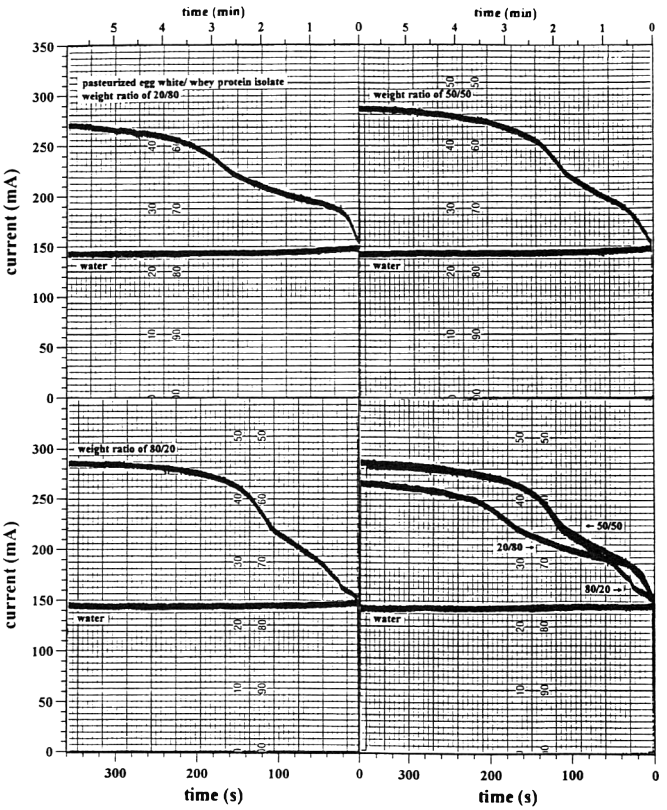


Fig. 4—Current input as related to whipping time for pasteurized egg white/whey protein isolate mixes.

rent input. With exception of total milk protein and soy protein isolate all other solutions of pure proteins tested could be distinguished by the maximum current input used in whipping them.

Compression tests were less sensitive in characterizing foam rigidity than were Cream Tester data (Fig. 5). Milk and soy protein isolate foams were difficult to characterize due to low strength and/or stability. Some foams broke down immediately after beating (skim milk powder at 5% TS, κ-caseinate at 5% TS and soy protein isolate) and could not be measured. For micellar casein, skim milk, total milk protein and κ-caseinate, no difference was found by Instron analysis (Table 1). Different batches of pasteurized egg white gave different results. A good correlation ($P \leq 0.001$) occurred between the Cream Tester and Instron data for the dilution series of pasteurized egg white.

Comparing the drainage rate of the different foams (Fig. 6) egg white foams were the most stable. The 50/50 (w/w) and 80/

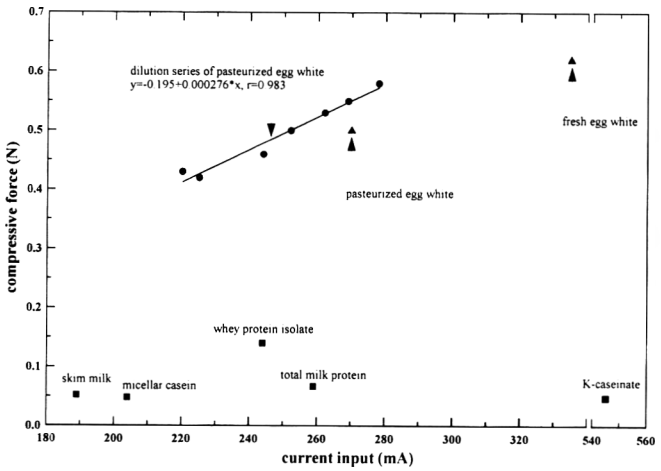


Fig. 5—Comparison of results of Cream Tester whipping and Instron analysis.

20 (w/w) mixes of pasteurized egg white and whey protein isolate showed a higher final current value. However, their drainage rate was between those of foams from the individual proteins. Foams of micellar casein, κ-caseinate at 5% TS and skim milk at 5% TS had very low stability. For κ-caseinate at 10% TS, the method is not applicable since the broken foam sticks to the sieve due to the high viscosity of the caseinate solution. The specific volume of all foams indicated relatively low overrun as compared to published data (Phillips et al., 1987). This may be due to different geometries and materials of the whipping system, different beater velocities or differences in proteins (Phillips et al., 1990). In our study sub-optimal foam expansion may be caused by too low energy input to the whipped solution. This may limit the applicability of the Cream Tester for low foaming products especially at lower concentrations.

The methods to determine specific volume and drainage were designed to avoid foam damage during preparation of samples. Therefore, results showed relatively low coefficients of variation (mean CV = 2.1% for specific volume and 4.7% for drainage). Foam rigidity, i.e., current input during beating, showed much smaller coefficients of variation (mean CV = 0.8%) than corresponding results from compression analysis (mean CV = 5.6%).

CONCLUSIONS

THE WHIPPING MACHINE proved extremely applicable to produce egg white foams and characterize their rigidity. The modification of whipping equipment and the adaptation of methods for foam characterization allowed a nondestructive handling of foams for more precise analysis. Application to milk proteins with high foaming capacity is possible. Maximum energy input of the machine cannot be increased so its use for low-foaming protein solutions would not be applicable.

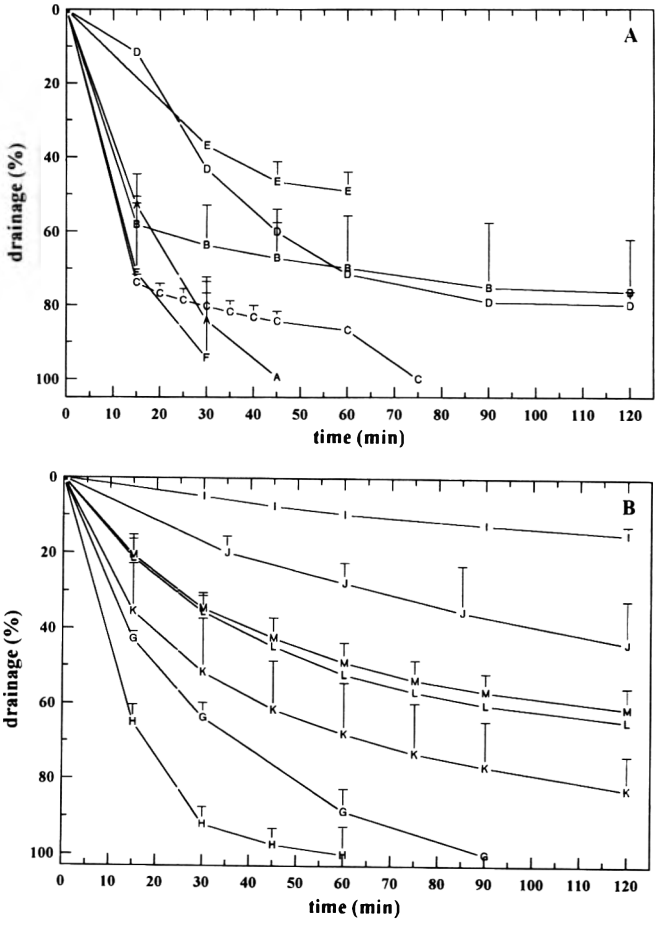


Fig. 6—Drainage rate of protein solutions (10% TS). (A) A: micellar casein, B: skim milk, C: skim milk at 5% TS, D: total milk protein, E: κ-caseinate*, F: κ-caseinate at 5% TS. (B) G: whey protein isolate, H: soy protein isolate, I: fresh egg white, J: pasteurized egg white, K: 20/80, L: 50/50, M: 80/20 weight ratio pasteurized egg white/whey protein isolate (* sticks to the sieve). Confidence intervals (95%) that were greater than the print size of the sample identification letters are given by error bars.

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Factors Affecting Light-Induced Pink Discoloration of Annatto-Colored Cheese

C.M. HONG, W.L. WENDORFF, and R.L. BRADLEY, JR.

ABSTRACT

The effects of light type, exposure and intensity, storage temperature, pH, fat content, and age and source of colorant were evaluated on stability of annatto colorants in cheese. Exposure to cool white fluorescent lighting resulted in a decrease in Hunter b values and hue throughout storage. Decreased temperature reduced the loss of a and b values after 2 days of light exposure. As time of exposure increased, reduction of a and b also increased. As pH changed from 5.4 to 4.8, b values were reduced at a faster rate than a values resulting in an intense pink color. Commercial cheese colorants extracted from annatto seeds were variable in color stability.

Key Words: annatto, discoloration, cheese, pink color

INTRODUCTION

DISCOLORATION OF FOOD PRODUCTS may reduce consumer acceptability of their overall quality. Pink discoloration of annatto-colored cheese is experienced in retail display conditions where cheeses may be exposed to high-intensity fluorescent lighting (Hong and Wendorff, 1993).

Hood and White (1929) reported on a series of studies on pink discoloration of colored cheeses in Canada. Pink discoloration of annatto-colored cheeses was also reported by Moir (1933) and Morgan (1933). They observed a result from mold enzymes and acids reacting with the annatto color under chemically reduced conditions. Barnicoat (1937, 1950) concluded that sulfhydryl compounds were responsible for oxidation of bixin, which resulted in pink discoloration. Govindarajan and Morris (1973) attempted to isolate and characterize pink compounds found in discolored Cheddar cheese. They reported that the pink compound contained norbixin, possibly protected by phospholipids and β-casein. They concluded that the pink compound was not a result of oxidation of norbixin, but that hydrogen sulfide was involved in the formation of a pink colored, microfine precipitate of norbixin.

Kristoffersen et al. (1964) were the first researchers to report that fluorescent light induced changes in cheeses. They described light-induced flavor changes in Cheddar cheese but did not report on color changes. Riddet et al. (1961) exposed Cheddar cheese curds to sunlight for 45 min and found no significant discoloration. Deger and Ashoor (1987) reported that color of Cheddar cheese faded at fluorescent light intensities of 5380 lux. They indicated that color of cheese may fade at light intensities of 1614 lux or higher. In a preliminary study, Hong and Wendorff (1993) observed pink discoloration on light-exposed surfaces of Cheddar and Colby cheese after < 48 hr storage under retail display conditions. Results of a market survey (Hong, 1994) indicated that all cheeses colored with annatto were susceptible to pink discoloration.

Our objective was to evaluate effects of light, pH, temperature, colorants, and fat content of cheese on the development of pink discoloration in annatto-colored cheese under retail display lighting.

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MATERIALS & METHODS

Cheese and annatto

Two-month-old Monterey Jack cheese was obtained from a commercial cheese plant and stored in the dark at 3°C until needed. Natural annatto cheese colorants were obtained from various commercial colorant suppliers and stored at 3°C. These colorants were standardized to the same α-norbixin concentration by adding aqueous 0.1 N KOH solution until the absorbance was the same as the weakest colorant, measured at 482 nm (Bausch and Lomb Model 20, Rochester, NY) (Reith and Gielen, 1971).

Preparation of colored cheese paste

Annatto-colored cheese paste was prepared by blending 240 g of cheese and 150 g distilled water in an Oster blender (John Oster Mfg. Co., Milwaukee, WI) at the following speeds until the paste was smooth: low for 90 sec, high for 30 sec, low for 30 sec, and high for 30 sec. Standardized single-strength annatto colorant was added to the cheese paste at 0.3% (v/v) and hand-mixed until color was uniform. Commercial annatto colorant A (Table 7) was used unless otherwise stated. Cheese paste pH was measured with the quinhydrone electrode (Van Slyke and Price, 1979) and was adjusted to 4.8, 5.1, and 5.4 with aqueous 0.1N NaOH or 0.5N lactic acid; cheese paste pH was 5.1 unless otherwise stated. In the high-fat cheese paste, 90 g of 30% cream was substituted for distilled water. The colored cheese paste was divided into three portions and packed in 15 × 60 mm plastic sterile Petri dishes, covered and sealed with transparent tape.

Light display and sampling methods

Samples of colored cheese paste were displayed at 8°C (unless otherwise stated) under 3500 lux of continuous lighting (40W cool white fluorescent unless otherwise stated). Samples were randomly located within the display and rotated systematically to assure uniform exposure to light. A duplicate set of initial samples was stored in the dark at 8°C. Color evaluations of each sample were made after 0, 2, 7, and 14 days storage.

Color evaluation

Color of samples was measured using a Hunter Lab D-25A-9 Tristimulus Colorimeter (Hunter Associates Laboratory, Inc., Reston, VA). The colorimeter was standardized with a white standard plate No. C2-18509 (X = 82.62; Y = 84.9; Z = 100.11), and the accuracy was checked with a pink reference plate No. C2-18510 (X = 54.6; Y = 46.8; Z = 41.9). Hunter color values were determined following the procedure of Riha and Wendorff (1993). Hunter L (lightness), a (red) and b (yellow) values were reported and hue angles (Θ) were calculated [(Θ) = 0 for red, (Θ) = 90 for yellow] (Francis, 1975; Little, 1975). All treatments were analyzed in duplicate.

Statistical analysis

Statistical analyses were conducted using Minitab Release 8 (Minitab, Inc., State College, PA). Differences between treatments were determined using one-way analysis of variance (ANOVA) and the post hoc Tukey's HSD pairwise test ($P<0.05$).

RESULTS & DISCUSSION

MEAN VALUES of Hunter L (lightness), a (redness), and b (yellowness) for light-induced pink discolored cheese were compared (Fig. 1). Hunter L increased slightly during storage under

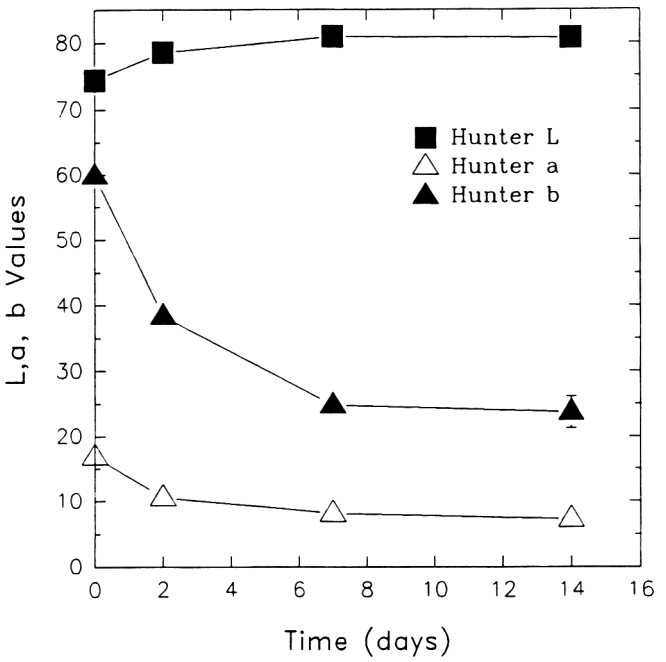


Fig. 1—Surface discoloration (Hunter L, a, b values) of annatto-colored cheese paste during storage under fluorescent light (3500 lux) at 8°C.

fluorescent light. Hunter b decreased at a rapid rate while Hunter a decreased slowly with time of exposure. The decrease in a and b values indicated the level of pink discoloration on the cheese surface was dependent on the ratio of red and yellow components remaining in the sample.

Type of lighting

Changes in a and b values of annatto-colored cheeses displayed under two types of retail lighting were compared (Table 1). Samples under cool white fluorescent light showed a greater ($P<0.05$) decrease in b values than under soft white fluorescent light. Those stored under cool white fluorescent light had lower ($P<0.05$) hue angles and more intense pink discoloration. Riddet et al. (1961) reported that cheese was not susceptible to light discoloration. However, our studies indicated that exposure to either type of fluorescent light resulted in measurable pink discoloration.

Light intensity

Data indicated the effect of light intensity on color stability (Table 2). Increased light intensity resulted in a ($P<0.05$) greater decrease in a and b values over the first 2 days of exposure but were not different with increased storage. Deger and Ashoor (1987) reported that the appearance of Cheddar and Colby cheeses did not change when stored 14 days at light intensities of 1614 lux or lower. They determined light intensities of 108 to 1937 lux in dairy cases in 10 local stores in Arizona. We observed light intensities in the range of 1722 to 5167 lux in dairy cases in the Madison, WI area (Hong, 1994).

Storage temperature

Samples stored at 8°C had greater ($P<0.05$) decreases in a and b values at 2 days than those stored at 2°C (Table 3). However, no significant difference ($P<0.05$) in color stability occurred between the two storage temperatures with storage more than 2 days.

Table 1—Effect of type of light[c] on color properties of annatto-colored cheeses under continuous lighting

Color property	Type of light	Days of storage 0	2	7	14
Lightness (L)	Cool white	76.85[a]	77.44[a]	76.95[a]	75.46[a]
	Soft white	76.06[b]	77.43[a]	76.41[b]	75.55[a]
Redness (+a)	Cool white	18.29[a]	14.23[a]	13.52[a]	12.41[a]
	Soft white	18.37[a]	14.62[a]	13.84[a]	12.46[a]
Yellowness (+b)	Cool white	59.88[a]	40.60[b]	33.25[b]	31.28[b]
	Soft white	60.21[a]	43.19[a]	35.93[a]	32.60[a]
Hue angle (Θ)	Cool white	73.02[a]	70.68[b]	67.87[b]	68.36[b]
	Soft white	73.04[a]	71.30[a]	68.89[a]	69.09[a]

[a-b] Means of a color property within a column with no common superscripts differ significantly ($P < 0.05$; $n = 8$).
[c] Cheeses were held at 8°C under fluorescent light intensity of 3500 lux.

Table 2—Effect of light intensity[c] on color properties of annatto-colored cheeses under continuous lighting

Color property	Light intensity, lux	Days of storage 0	2	7	14
Lightness (L)	3230	74.41[a]	78.54[b]	81.06[b]	81.72[a]
	6900	74.29[a]	80.67[a]	81.97[a]	81.89[a]
Redness (+a)	3230	16.84[a]	10.54[a]	7.40[a]	6.58[a]
	6900	16.88[a]	7.73[b]	6.53[a]	5.92[a]
Yellowness (+b)	3230	59.88[a]	38.33[a]	24.80[a]	23.21[a]
	6900	59.14[a]	27.43[b]	23.66[a]	21.49[a]
Hue angle (Θ)	3230	74.29[a]	74.63[a]	73.47[a]	74.18[a]
	6900	74.06[a]	74.26[a]	74.58[a]	74.60[a]

[a-b] Means of a color property within a column with no common superscripts differ significantly ($P < 0.05$; $n = 8$).
[c] Cheeses were held at 3°C under cool white fluorescent light.

Table 3—Effect of storage temperature[c] on color properties of annatto-colored cheeses uncer continuous lighting

Color property	Storage temp °C	Days of storage 0	2	7	14
Lightness (L)	2	74.43[a]	78.24[b]	81.28[a]	81.98[a]
	8	74.38[a]	78.54[a]	81.06[a]	81.72[a]
Redness (+a)	2	16.63[a]	11.24[a]	7.55[a]	7.31[a]
	8	16.83[a]	10.54[b]	7.40[a]	6.58[a]
Yellowness (+b)	2	59.90[a]	40.33[b]	25.98[a]	23.84[a]
	8	59.89[a]	38.33[b]	24.80[a]	23.21[a]
Hue angle (Θ)	2	74.24[a]	74.42[a]	73.80[a]	73.08[a]
	8	74.30[a]	74.63[a]	73.47[a]	74.10[a]

[a-b] Means of a color property within a column with no common superscripts differ significantly ($P < 0.05$; $n = 8$).
[c] Cheeses were exposed to cool white fluorescent light intensity of 3500 lux.

Time of exposure

Continuous exposure to light resulted in a decrease ($P<0.05$) of a and b values compared to a nonexposed control (Table 4). Exposure times of 6 hr or more resulted in decreases in a values (red) which increased hue angles with storage.

pH of cheese

The effect of cheese pH on color stability was determined (Table 5). At pH 5.4, the a value (red) decreased while the b value (yellow) also decreased, but at a slower rate than at pH 4.8 or 5.1. At pH 4.8 and 5.1, the decrease in b value was greater than at pH 5.4; however, the a value decreased at a slower rate. The result was a decrease ($P<0.05$) in hue angles and an intense pink color. A similar finding was noted by Barnicoat (1937) who observed that an annatto solution was reddish at pH 4.8 and yellowish-red at pH 5.7. Barnicoat also indicated that highly acidic cheese developed a deeper pink color than cheeses with normal pH.

Table 4—Effect of time of exposure[e] on color properties of annatto-colored cheess under continuous lighting

Color property	Time of exposure, hr	Days of storage 0	2	7	14
Lightness (L)	0	74.83[a]	75.05[d]	75.43[d]	75.49[d]
	6	74.73[a,b]	76.36[c]	76.42[c]	76.32[c]
	24	74.69[b]	77.97[b]	77.98[b]	77.94[b]
	Continuous	74.83[a]	80.16[a]	82.82[a]	83.74[a]
Redness (+a)	0	17.19[a]	17.38[a]	17.38[a]	17.39[a]
	6	17.07[a]	13.49[b]	13.42[b]	13.51[b]
	24	16.95[a]	10.32[c]	10.08[c]	9.27[c]
	Continuous	16.86[a]	8.44[d]	5.82[d]	4.75[d]
Yellowness (+b)	0	57.17[a]	56.91[a]	55.49[a]	55.93[a]
	6	56.29[a]	50.77[b]	51.03[b]	50.04[b]
	24	56.34[a]	39.57[c]	40.86[c]	40.76[c]
	Continuous	56.29[a]	31.16[d]	19.98[d]	19.33[d]
Hue angle (Θ)	0	73.26[a]	73.02[b]	72.60[d]	72.73[c]
	6	72.84[a]	75.12[a]	75.26[b]	74.89[b]
	24	73.25[a]	75.39[a]	76.14[a]	75.86[a]
	Continuous	73.32[a]	74.84[a]	74.57[c]	76.20[a]

[a-d] Means of a color property within a column with no common superscripts differ significantly ($P < 0.05$; $n = 8$).
[e] Cheeses were held at 8°C under cool white fluorescent light intensity of 3500 lux; after exposure, stored in dark at 8°C.

Table 5—Effect of pH of cheese[d] on color properties of annatto-colored cheeses under continuous lighting

Color property	pH of cheese	Days of storage 0	2	7	14
Lightness (L)	4.8	78.93[a]	80.76[a]	83.35[a]	84.57[a]
	5.1	77.07[b]	76.83[c]	79.28[c]	81.55[c]
	5.4	74.39[c]	79.26[b]	81.71[b]	83.20[b]
Redness (+a)	4.8	13.68[c]	12.69[b]	12.72[a]	9.07[a]
	5.1	14.83[b]	14.66[a]	12.39[a]	9.57[a]
	5.4	17.96[a]	11.26[c]	9.51[b]	7.85[b]
Yellowness (+b)	4.8	52.04[c]	35.50[a]	20.86[c]	13.98[c]
	5.1	55.27[a]	36.49[a]	23.75[b]	16.48[b]
	5.4	54.17[b]	35.99[a]	27.70[a]	22.83[a]
Hue angle (Θ)	4.8	75.27[a]	70.33[b]	64.48[b]	57.04[c]
	5.1	74.98[a]	68.08[c]	62.45[b]	59.87[b]
	5.4	71.65[b]	72.63[a]	71.03[a]	71.01[a]

[a-c] Means of a color property within a column with no common superscripts differ significantly ($P < 0.05$; $n = 8$).
[d] Cheeses were held at 8°C under cool white fluorescent light intensity of 3500 lux.

Table 6—Effect of fat content of cheese[c] on color properties of annatto-colored cheeses under continuous lighting

Color property	Fat content of cheese, g/100 g	Days of storage 0	2	7	14
Lightness (L)	18	75.51[b]	80.43[b]	82.70[b]	83.27[b]
	30	77.74[a]	82.10[a]	84.07[a]	84.57[a]
Redness (+a)	18	15.22[a]	7.82[a]	5.92[a]	5.24[a]
	30	14.20[b]	7.12[b]	5.27[b]	4.49[b]
Yellowness (+b)	18	51.10[a]	30.22[a]	21.12[a]	20.38[a]
	30	58.90[b]	27.97[b]	18.45[b]	18.76[b]
Hue angle (Θ)	18	73.41[b]	75.48[a]	74.35[a]	75.60[a]
	30	76.45[a]	75.72[a]	74.06[a]	76.54[a]

[a,b] Means of a color property within a column with no common superscripts differ significantly ($P < 0.05$; $n = 8$).
[c] Cheeses were held at 8°C under cool white fluorescent light intensity of 3500 lux.

Fat content of cheese

High-fat cheese showed lower ($P<0.05$) a and b values than low-fat cheese during storage under light (Table 6). However, both cheeses had comparable hue angles with lighted storage. Both Barnicoat (1937) and Riddet et al. (1961) noted that fat oxidation was critical to the discoloration of cheeses. Govindarajan and Morris (1973) reported that when extracting the pink color from discolored cheese, the pink color was associated with the fat layer. However, no specific information was reported on the possible importance of fat in relation to cheese discoloration.

Table 7—Effect of commercial annatto colorant[e] on color properties of annatto-colored cheeses under continuous lighting

Color property	Colorant sample	Days of storage 0	2	7	14
Lightness (L)	A	76.85[b]	77.44[a]	76.95[a]	75.46[b]
	B	75.38[d]	76.16[c]	75.62[c]	74.37[c]
	C	77.37[a]	77.52[a]	77.04[a]	76.49[a]
	D	76.02[b]	76.67[b]	75.78[c]	75.20[b,c]
	E	76.52[b]	77.26[a]	76.53[b]	74.75[c]
Redness (+a)	A	18.28[b]	14.23[c]	13.52[c]	12.41[c]
	B	19.52[a]	15.86[a]	15.42[a]	14.16[a]
	C	15.21[d]	12.35[d]	11.39[d]	10.18[d]
	D	17.40[c]	14.98[b]	14.27[b]	13.06[b]
	E	17.92[b]	15.18[b]	14.36[b]	13.60[b]
Yellowness (+b)	A	59.88[a]	40.60[a]	33.25[a,b]	31.28[a]
	B	57.58[b]	39.28[a,b]	33.69[a]	31.86[a]
	C	50.96[d]	38.96[b]	31.85[b]	29.27[b]
	D	53.04[c]	36.44[c]	31.77[b]	30.78[a,b]
	E	53.24[c]	36.70[c]	31.22[b]	29.84[b]
Hue angle (Θ)	A	73.02[a]	70.68[b]	67.87[b]	68.36[b]
	B	71.28[c]	68.00[c]	65.38[c]	66.01[c,d]
	C	73.38[a]	72.41[a]	70.31[a]	70.81[a]
	D	71.82[b]	67.64[c]	65.80[c]	67.00[c]
	E	71.40[b,c]	67.53[c]	65.30[c]	65.50[d]

[a-d] Means of a color property within a column with no common superscripts differ significantly ($P < 0.05$; $n = 8$).
[e] Cheeses were held at 8°C under cool white fluorescent light intensity of 3500 lux.

Table 8—Effect of source of annatto colorant[e] on color properties of annatto-colored cheeses under continuous lighting

Color property	Source of annatto	Days of storage 0	2	7	14
Lightness (L)	Wet bixin	73.10[b,c]	74.69[b]	75.21[b]	76.12[c]
	Seed F	73.39[b]	74.98[a,b]	75.66[a,b]	76.61[b]
	Seed G	73.78[a]	75.13[a]	76.26[a]	77.29[a]
	Seed H	73.40[b]	74.82[a,b]	76.14[a]	77.01[a,b]
	Seed I	73.01[c]	73.79[c]	75.86[a]	76.12[c]
Redness (+a)	Wet bixin	15.34[c]	15.30[c]	13.24[b]	10.66[c]
	Seed F	16.93[b]	16.42[a,b]	14.15[a,b]	11.53[b]
	Seed G	16.56[b]	15.96[b]	13.70[b]	11.22[b]
	Seed H	18.28[a]	16.76[a]	14.65[a]	12.14[a]
	Seed I	18.29[a]	16.54[a]	14.46[a]	12.21[a]
Yellowness (+b)	Wet bixin	46.66[b]	31.82[d]	25.54[d]	22.96[d]
	Seed F	54.84[a,b]	37.06[b]	28.56[b]	24.84[b]
	Seed G	49.68[b]	33.98[c]	26.79[c]	23.97[c]
	Seed H	55.80[a,b]	37.24[b]	29.02[b]	25.59[a]
	Seed I	56.56[a]	38.49[a]	29.86[a]	26.17[a]
Hue angle (Θ)	Wet bixin	71.80[a,b]	64.31[c]	62.60[b]	65.10[a]
	Seed F	71.66[a,b]	66.08[a,b]	63.64[a,b]	65.09[a]
	Seed G	71.56[b]	64.84[c]	62.92[b]	64.92[a]
	Seed H	71.87[a,b]	65.77[b]	63.22[b]	64.63[a]
	Seed I	72.08[a]	66.74[a]	64.17[a]	64.99[a]

[a-d] Means of a color property within a column with no common superscripts differ significantly ($P < 0.05$; $n = 8$).
[e] Cheeses were held at 8°C under cool white fluorescent light intensity of 3500 lux.

Annatto colorant

The commercial sources of annatto colorants were related to some differences ($P<0.05$) in color stability under fluorescent lighting (Table 7). Colorant sample A showed the greatest loss in a and b values. Colorant C showed the lowest tendency for pinking with the highest hue angles.

The effect of source of anatto seeds on color stability was evaluted (Table 8). Results indicated some variation in color stability between different sources of annatto seed. The wet bixin sample had lower color values initially but was comparable to seed samples in hue angles.

As the annatto colorant aged, the b value was lower and the a value higher than in fresh colorant (Table 9). The 1-month-old colorant was more susceptible to loss of yellow component than was the aged colorant. The aged colorant showed a greater tendency for pink discoloration than the fresh colorant. Reith and Gielen (1971) reported that an old annatto solution exposed to light contained less yellow and red pigments than fresh annatto samples after exposure. They also concluded that aged

Table 9—Effect of age of colorant[c] on color properties of annatto-colored cheeses under continuous lighting

Color property	Age of colorant, month	Days of storage			
		0	2	7	14
Lightness (L)	1	73.10[b]	74.69[b]	75.21[b]	76.12[b]
	12	75.96[a]	77.91[a]	78.41[a]	79.95[a]
Redness (+a)	1	14.47[b]	11.74[b]	10.49[b]	9.50[b]
	12	15.56[a]	12.82[a]	11.52[a]	10.33[a]
Yellowness (+b)	1	53.19[a]	28.50[a]	20.83[a]	19.72[a]
	12	48.78[b]	28.75[a]	21.41[a]	20.28[a]
Hue angle (Θ)	1	74.78[a]	67.57[a]	63.28[a]	64.29[a]
	12	72.92[b]	65.94[b]	61.72[b]	63.02[b]

[a-bb,c] Means of a color property within a column with no common superscripts differ significantly ($P < 0.05$; $n = 8$).

[c] Cheeses were held at 8°C under cool white fluorescent light intensity of 3500 lux.

colorant had less α-norbixin content. Barnicoat (1937) also reported that older samples of annatto colorants contained some polymerized residues of norbixin. Apparently, changes in chemical composition may result in stability problems when using aged colorants.

CONCLUSIONS

PINK DISCOLORATION of annatto-colored cheese under fluorescent lighting was affected by type of lighting, storage conditions, cheese composition, and source of colorant. The pH of cheese had a strong influence on the pattern of discoloration. Low-pH cheese had a rosy pink discoloration and higher-pH cheese had a pale pink discoloration. Further studies are needed to determine if packaging or changes in lighting would be effective in reducing the pink discoloration of annatto-colored cheeses in supermarket dairy display cases.

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Microencapsulation by Spray Drying Ethyl Caprylate in Whey Protein and Carbohydrate Wall Systems

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ABSTRACT

Ethyl caprylate was microencapsulated by spray drying in wall systems consisting of whey protein in combination with maltodextrin (DE 5-15) or corn syrup solids (DE 24). Ester particle-size distribution in the infeed emulsion was adversely affected by high proportions of low DE maltodextrin. Ester retention during drying was affected ($P < 0.05$) by type of carbohydrate used and its proportion and ranged from 70 to 91%. The proportion of solvent extractable core was reduced with increasing DE value of carbohydrate. Combinations of whey proteins and high DE maltodextrins or corn syrup solids are effective wall systems for microencapsulation of volatiles.

Key Words: microencapsulation, whey proteins, maltodextrins, volatiles

INTRODUCTION

MICROENCAPSULATION IS A TECHNIQUE by which liquid droplets or solid particles are coated with a thin film of protective materials. The microencapsulated materials (core) are covered by a film forming (or coating) material (wall or shell, Dziezak, 1988; Jackson and Lee, 1991; Shahidi and Han, 1993). The film or wall system protects the core against deterioration, limits evaporation of volatile core, and releases the core under desired conditions (Shahidi and Han, 1993). Fats and oils, aroma compounds and oleoresins, vitamins, minerals, colorants, and enzymes have been microencapsulated (Dziezak, 1988; Jackson and Lee, 1991; Shahidi and Han, 1993). Spray drying is the most common microencapsulation technique in the food industry (Dziezak, 1988, Jackson and Lee, 1991; Reineccius, 1988; Versic, 1988).

The functionality profile of wall materials for spray drying includes high solubility effective emulsification and film forming characteristics, and efficient drying properties. In addition, concentrated solutions of wall material should have low viscosity (Reineccius, 1988). Whey proteins have such properties and were reported as an effective basis for microencapsulation by spray drying of anhydrous milkfat or volatiles (Moreau and Rosenberg, 1993; Rosenberg and Young, 1993; Sheu and Rosenberg, 1993; Young et al., 1993a,b).

Maltodextrins have been used in microencapsulation of food components (Kenyon and Anderson, 1988; Shahidi and Han 1993). Maltodextrins and low dextrose-equivalence (DE) corn syrup when dried, manifest matrix forming properties important in wall systems. (Kenyon and Anderson, 1988). However, the surface active characteristics of such carbohydrates, and the low viscosity of their solutions do not promote emulsification of oil-like materials. A stable emulsion of fine droplets of core material in the wall solution is critical in microencapsulation (Kenyon and Anderson, 1988; Risch and Reineccius, 1988). When maltodextrins or corn syrup solids are used as wall constituents it is thus necessary to incorporate other wall materials with improved emulsifying characteristics.

Retention of volatile core material during microencapsulation by spray drying is affected by chemical and physical properties of the wall and core materials (Bomben et al, 1973; Leahy et al., 1983; Menting et al., 1970; Reineccius, 1988; Reineccius and Coulter, 1969; Rosenberg, 1985; Rosenberg et al., 1990; Rulken and Thijssen, 1974), properties of the infeed (Reineccius, 1988), and atomization and drying conditions (Reineccius, 1988). The microstructural features of the capsules affect volatile retention during storage (Chang et al., 1988; Rosenberg, 1985; Rosenberg et al., 1985, 1988).

Bangs and Reineccius (1981) reported that retention of volatile flavor compounds decreased as maltodextrin DE increased. High DE maltodextrins protected encapsulated orange peel oil against oxidation (Anandaraman and Reineccius, 1986) suggesting the importance of DE to the functionality of the wall system. Limited information suggesting the advantage of using mixtures of dextrin and proteins for microencapsulation of volatiles has been reported by Bangs and Reineccius (1981). A mixture of gum arabic and maltodextrin was reported effective in microencapsulation of cardamon oil (Sankarikutty et al., 1988). Mixtures of whey proteins with natural or modified carbohydrates were used for microencapsulation of anhydrous milkfat (Young et al., 1993b). In such systems, whey proteins served as emulsifying and film-forming agent while the carbohydrates acted as a matrix-forming material. Mixtures of whey proteins and maltodextrins thus appear to provide the functionality profile for successful microencapsulation of volatile materials.

Our objective was to study microencapsulation by spray drying of a volatile component in wall systems consisting of mixtures of whey proteins and maltodextrins (of different DE) or corn syrup solids. The effect of carbohydrate type, and its proportion on emulsion characteristics, volatile retention and solvent extractability were included.

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MATERIALS & METHODS

Wall and core materials

Whey protein isolate (WPI) was purchased from Le Sueur Isolates (Le Sueur, MN). Maltodextrins of 5, 10, and 15 DE (designated: MD5, MD10, MD15, respectively) and 24 DE corn syrup solids (CSS) were purchased from American Maize-Products Company (Hammond, IN). Ethyl caprylate (ECY) was purchased from Sigma Chemical Company (St. Louis, MO).

Microencapsulation

Emulsion preparation. A wall solution containing 25% (w/w) solids consisting of WPI, or WPI-carbohydrate combination were prepared in 25°C deionized water and cooled to 4°C. WPI-carbohydrate (WPI/COH) weight ratios of 1:19, 1:9, 1:1, or 3:1 were used. The model ester (at 4°C) was emulsified into the wall solutions at a proportion of 30% (w/w of wall solids). Emulsification was carried out by first preparing a coarse emulsion using an Ultra-Turrax® T-25-S1 homogenizer (IKA Works, Cincinnati, OH) operated at 13,500 rpm for 30 sec followed by four successive homogenization steps using a Mini-Lab high pressure homogenizer (type 8.30H; Rannie, St. Paul, MN) operated at 50 MPa. The emulsion was maintained at 15°C during homogenization. The final emulsion was kept at 5°C until used. Four ethyl caprylate emulsions stabilized only by WPI at 1.25, 2.5, 12.5, and 18.75% w/w were also prepared as described. The WPI concentration in these emulsions was identical to that in each of the WPI/COH stabilized emulsions. Ethyl caprylate load in each of these emulsions was identical to that used in the 25% solids samples.

Spray drying. Emulsions were spray dried using an APV Anhydro Laboratory Spray Dryer (APV Anhydro A/S. Søborg, Denmark). The dryer had an evaporation rate of 7.5 kg/h and a chamber diameter of 1 m. The centrifugal atomizer of the dryer was used at 50,000 rpm and drying was in the concurrent drying configuration. Based on a previous study (Sheu and Rosenberg, 1993) feed at 5°C, inlet air at 160°C, and outlet air at 80°C were used in all cases. The collected dry powders were kept in a desiccator at 25°C. All experiments were carried out in duplicate.

Analyses

Emulsion particle size distribution. Droplet-size distribution in the emulsions was determined using a Malvern Mastersizer MS20 (Malvern Instruments, Malvern, Worcs., UK). A 2 mW He-Ne laser beam (633 nm) and a 45 mm focus lens were used. The volume-size average d_{32} was used as a particle size distribution parameter.

Surface tension

Surface tension of some wall solutions was determined using a Fisher Surface Tensiomat (Model 21, Fisher Scientific Co., Pittsburgh, PA). Measurements were carried out in triplicates at 25°C using a platinum iridium ring (R/r=53.0322424).

Chemical analyses

Moisture content of the microcapsules was determined gravimetrically (in triplicates) after 4 hr vacuum drying (40°C, 3 kPa). If needed, weight corrections for volatile loss were made from ester content prior to and following vacuum drying.

Ester content of the dry microcapsules was determined using a modification of the method described by Rosenberg et al. (1990). One gram of powder was dissolved in 100 mL of deionized water at 25°C. After 15 min incubation at 25°C, a 0.5 mL aliquot of emulsion was mixed with 4.5 mL 50% ethanol solution. Then, 4 mL of a fresh reagent mixture consisting of a 1:1 ratio of 3N sodium hydroxide and 13.9% hydroxylamine was added, the mixture was thoroughly mixed, and incubated for 10 min at 25°C. 2 mL of 3N hydrochloric acid was then added to stop the reaction. The color was developed after addition of 2 mL 10% ferric chloride solution in 0.1N hydrochloric acid and measured by absorbance at 525 nm. The amount of ester was determined from calibration standard curves.

A preliminary study was carried out to verify that the ester content determined by the method represented volatiles that were truly encapsulated. Samples of ethyl caprylate containing microcapsules were washed for 30 s with petroleum ether and recovered (using the method described below) for the determination of microencapsulation efficiency. The washed microcapsules were dried for 4 hr under vacuum (40°C, 3 kPa) and then placed in a desiccator (25°C). The ester content of treated capsules was determined using the described methods and compared to that of microcapsules (from the same batch) that had not been washed and dried.

Petroleum ether extractable ester was determined as described by Young et al. (1993a). One gram of microcapsules was extracted with 10 mL of petroleum ether in a 30 mL hermetically sealed extraction flask for 5 min. at 25°C. The mixture was filtered through a GN-6 filter (0.45 μm; Gelman Science, Inc., Ann Arbor, MI). One mL of filtrate was mixed with 4 mL of 95% ethanol and the ester determination was used. All ester determinations were carried out in triplicate.

The ratio (as percentage) of encapsulated ester to initial ester in the emulsion, dry solids basis, was defined as the retention. The percentage of encapsulated ester that could not be extracted from 1g of dry microcapsules was designated the microencapsulation efficiency (EE).

Scanning electron microscopy (SEM)

Scanning electron microscopy studies of the inner and outer structural features of the dry microcapsules were carried out using the methods described by Rosenberg and Young (1993) and by Young et al. (1993a, 1993b). For examining their outer structure, microcapsules were attached to SEM stubs using a two-sided adhesive tape (Ted Pella, Redding, CA). The method described by Rosenberg and Young (1993) was used to examine the inner structure of the capsules. Microcapsules were fractured by moving a razor blade perpendicularly through a layer of capsules attached to the specimen holder by two-sided adhesive tape. Specimens were subsequently coated with gold using a model E-50050 Polaron Sputter Coater (Bio-Rad, San Jose, CA), and analyzed using an ISI DS-130 scanning electron microscope (International Scientific Instrument, Inc., Pleasanton, CA) operated at 10 kV. Type 55 Polaroid film (Polaroid Corp., Cambridge, MA) was used to produce micrographs.

Statistical analyses

The significance ($P < 0.05$) of experimental results was evaluated by ANOVA, Student-Newman-Keuls, and t-test procedures using the SigmaState™ software (Jandel Scientific, San Rafael, CA).

RESULTS & DISCUSSION

DISTRIBUTIONS OF ECY PARTICLE SIZES in emulsions of various wall solutions were compared (Fig. 1). Specific surface area and volume-surface average droplet diameter (d_{32}) of the emulsions were also compared (Table 1). Both WPI-carbohydrate (WPI/COH) ratio and carbohydrate DE affected particle-size distribution.

Results indicated an effect ($P < 0.05$) of WPI/COH ratio on d_{32} in emulsions consisting of WPI and MD5 or MD10. At WPI/COH ratios of 1:19 and 1:9 d_{32} was larger than that in emulsions with a WPI/COH ratio of 1:1 or 3:1 (Fig. 1A and 1B), and larger than that in WPI/ECY emulsion (0.43 μm). ECY particle size distributions in emulsions with a WPI/COH ratio of 1:1 or 3:1 were similar to each other and to that in WPI/ECY emulsion.

Particle-size distribution in emulsions consisting of MD15 or CSS was not affected by WPI/COH ratio (Fig. 1C and 1D and Table 1) and was similar ($p > 0.05$) to that in WPI/ECY emulsions.

At WPI/COH ratios of 1:19 and 1:9, differences ($P < 0.05$) were observed between emulsions containing MD5 or MD10 and those containing MD15 or CSS; however, no differences were observed within each of these two groups. ECY particle size distribution was not affected by carbohydrate DE in emulsions with a WPI/COH ratio of 1:1. At a WPI/COH ratio of 3:1, ECY particle size distributions of emulsions containing MD10, MD15, or CSS were similar to each other and differed ($P < 0.05$) from that in the MD5-containing emulsion.

None of the carbohydrates had surface active properties and thus we could assume that there was no competition between the whey proteins and carbohydrates at the o/w interface. The d_{32} of ECY emulsions stabilized by WPI at concentrations identical to those in the WPI/COH mixtures was 0.56, 0.50, 0.40, and 0.39 μm for emulsions stabilized by 1.25%, 2.5%, 12.5%, and 18.75% WPI, respectively. Results indicated that the absolute amount of WPI incorporated into the WPI/COH stabilized emulsion could emulsify the ECY. These findings suggested that the effect of WPI/COH on d_{32} in the WPI/MD5 or the WPI/MD10 emulsions, and especially where high proportions of carbohydrate were used was not likely to represent the result of a WPI concentration that was too low.

Interactions between proteins and carbohydrates that adversely affect particle-size distribution in emulsions have been reported (Dickinson and Euston, 1991; Dickinson and Galazka, 1991). Such interactions could have been the reason for the differences in d_{32} observed among-emulsions. This was evaluated only to a limited extent by comparing the surface tension of wall systems consisting of the WPI and MD5 mixtures to that of a WPI-based solution (prepared and measured under the same conditions). Surface tension values of 57.1, 55.3, 53.9, and 50.3 dyne/cm were observed for solutions consisting of WPI/MD5 at a ratio of 1:19, 1:9, 1:1, and 3:1, respectively. The surface tension of a WPI solution was 49.3 dyne/cm. These results suggest interactions may occur between MD5 and whey proteins that alter the emulsification properties of the wall system. Comparing the surface tension of each of the WPI/MD5 mixtures with that of the WPI solution indicated differences ($P < 0.05$) only up to a WPI/COH ratio of 1:1. These results were in agreement with our previous results regarding the d_{32} of these systems. They suggest that the molecular weight (and size) of the carbohydrate influenced the suggested protein-carbohydrate interactions. No

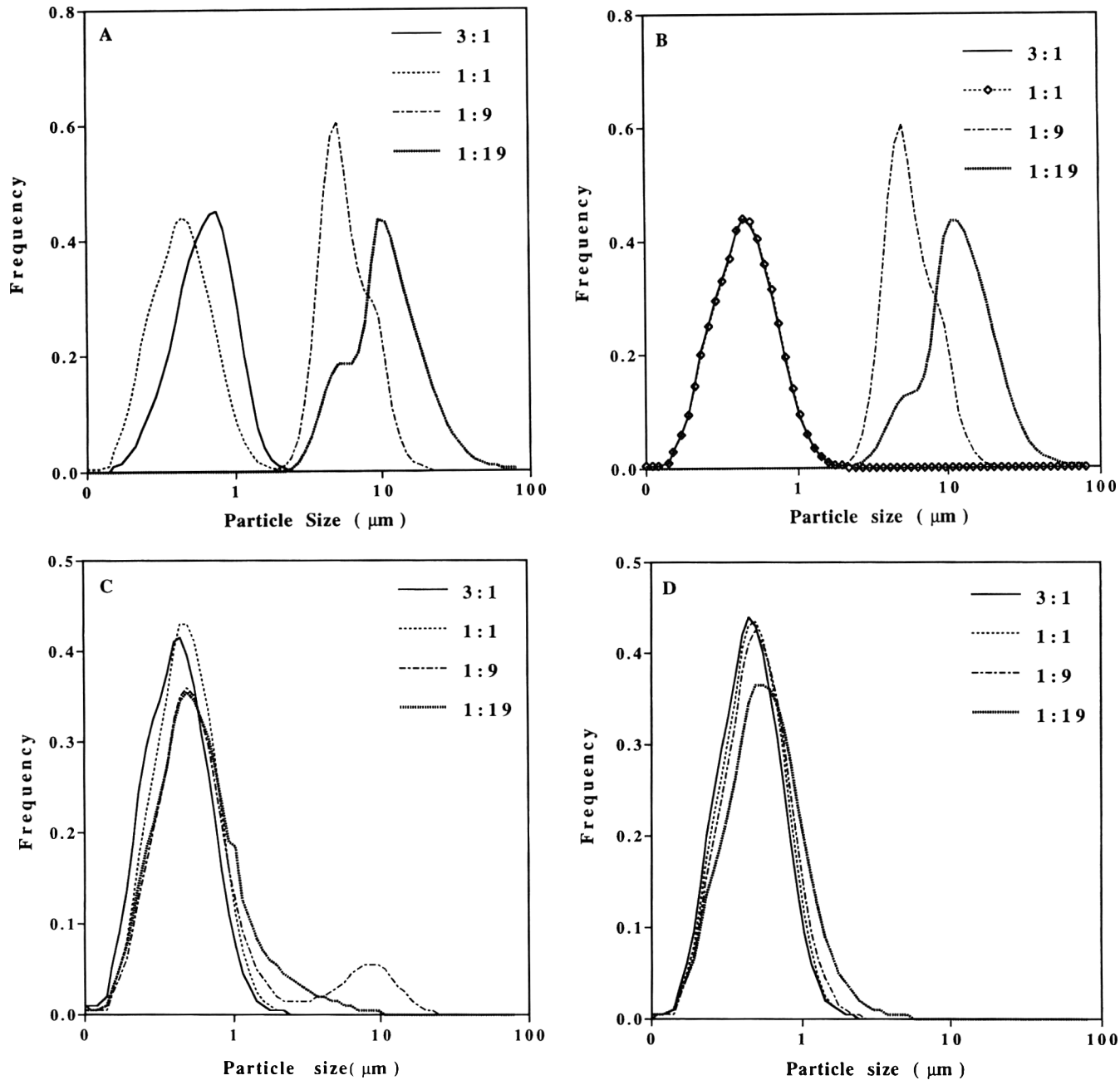


Fig. 1—Particle-size distribution of ethyl caprylate emulsions stabilized by different combinations of WPI and MD5 (A); MD10 (B); MD15 (C); and CSS (D). Total solids of continuous phase 25% (w/w) and ester load 30% (w/w of wall solids). Whey proteins-to-carbohydrate ratios were 1:19, 1:9, 1:1, and 3:1.

Table 1—Mean particle size (d_{32}) and specific surface area of ethyl caprylate (ECY) emulsified in wall solutions consisting of mixtures of WPI and various carbohydrates (Ester load 30% (w/w of wall solids) and wall solids 25% (w/w))

	Ratios of WPI to carbohydrates							
	1:19		*1:9*		*1:1*		*3:1*	
	d[v]	A[w]	d	A	d	A	d	A
Maltodextrin (DE = 5)	8.71[a]	0.70[x]	5.07[b]	1.19[x]	0.37[c]	16.4[y]	0.53[c]	11.6[z]
Maltodextrin (DE = 10)	9.94[a]	0.62[x]	5.05[b]	1.20[x]	0.39[c]	15.81[y]	0.39[c]	16.0[y]
Maltodextrin (DE = 15)	0.46[a]	13.50[x]	0.48[a]	13.01[x]	0.41[a]	15.07[x]	0.36[a]	17.23[x]
Corn syrup solids (DE = 24)	0.46[a]	13.31[x]	0.42[a]	14.50[x]	0.41[a]	15.1[x]	0.39[a]	15.80[x]

[a,b,c] d_{32} means within same row followed by different letters significantly different ($P < 0.05$).
[v] d_{32} mean values (μm).
[w] Specific surface area mean values (m^2/mL).
[x,y,z] Specific surface area means within same row followed by different letters significantly different ($P < 0.05$).

further attempt was made to investigate the nature of such interactions.

Ester retention

In a preliminary study, the ethyl caprylate content of petroleum ether-washed and vacuum-dried microcapsules was compared to that of unwashed capsules (from the same batch). In all cases, the washed capsules had ester content only slightly lower (by up to 10%) than that of the unwashed ones. These differences could be attributed to the removal of surface ester by the solvent, to some ester loss due to effects of vacuum drying, or to extraction of some of the encapsulated ester by the

solvent (Sheu and Rosenberg, 1993, Young et al., 1993a, 1993b). The high solubility of ethyl caprylate in petroleum ether suggests that if a large proportion of ester was unencapsulated, it should have resulted in larger differences in ester content between washed and unwashed capsules. Results thus indicated that the ester content mainly represented truly encapsulated material.

The retention of ECY in the various wall systems after microencapsulation by spray drying was compared (Table 2). The highest retention level (91.35%) was exhibited by wall systems consisting of WPI/MD10 (1:9) and was similar ($P > 0.05$) to that obtained by wall systems consisting of WPI/MD10 (1:19), WPI/MD15 (1:9), and WPI/CSS (1:9 and 3:1). All other wall systems exhibited lower retention levels ($P < 0.05$). Retention levels obtained by using a wall system consisting of a 25% WPI solution (71.85%) were compared with that obtained by using the various WPI/COH systems. They revealed that except for three systems (WPI/MD5 1:19, and 1:9; WPI/MD10 1:1), the retention level of the WPI-based system was lower ($P < 0.05$) than that with the WPI/COH-based systems.

Increasing the proportion of WPI in wall systems containing MD5 increased ($P < 0.05$) ester retention (Table 2). However, retention levels for the WPI/MD5 systems with a WPI/COH ratio of 1:1 or 3:1 were similar to each other. Risch and Reineccius (1988) suggested that retention of volatiles during microencapsulation by spray drying could be enhanced by reducing the mean particle size of the dispersed core material during emulsification. This, and the observations regarding the d_{32} of the WPI/MD5 emulsions, suggest that ECY retention was improved (in part) by reducing the d_{32} of ECY.

Ester retention in wall systems containing MD15 or CSS was less affected by WPI/COH ratio than that in wall systems containing MD5 or MD10 (Table 2). This was in agreement with our findings regarding the mean particle size in these emulsions and may thus provide additional evidence for the importance of d_{32} in affecting volatile retention in WPI/COH systems. This may also support the observation of Anandraman and Reineccius (1986) regarding the effect of DE value on film forming properties of maltodextrins.

Differences in ester retention in the various wall systems could not be explained based only on differences in ECY particle size-distribution. Ester retention in most WPI/COH wall systems was higher than that exhibited by a WPI-based wall system although the d_{32} of some systems (such as WPI/MD10 1:19) was larger ($P < 0.05$) than that of the WPI-based system. In other cases, although no differences were observed between the d_{32} of the WPI-based system and the WPI/COH systems, the retention level with the WPI-based system was lower. Thus incorporating carbohydrates into the wall systems improved the drying properties of the wall matrix and probably enhanced the formation of a dry crust around the drying droplets. Faster formation of a dry crust enhanced volatile retention during microencapsulation by spray drying (Reineccius, 1988; Rosenberg, 1985; Rosenberg et al., 1990).

Volatiles retention in maltodextrins has been reported to decrease with increasing DE values (Bangs and Reineccius, 1981). Our results did not show this effect and no trend characterizing effects of maltodextrin DE value on ECY retention could be identified. Results suggest that ester retention in WPI/maltodextrin- or CSS-containing wall systems was probably determined by both the effect of carbohydrate on emulsification properties of whey proteins and the overall drying characteristics of the wall systems. Both these factors were affected by the carbohydrate DE and its proportion in the wall system. The differences among carbohydrates in ester retention that we observed at a given WPI/COH ratio could be attributed to the extent to which each carbohydrate affected emulsion characteristics and drying properties.

Using a high wall solids concentration enhanced volatile retention during microencapsulation by spray drying (Reineccius, 1988). High retention levels of the ester, comparable and in most cases higher than those obtained with 30% gum arabic (Rosenberg et al., 1990). occurred at 25% (w/w) wall solids concentration. This indicated the effectiveness of whey protein/carbohydrate-based wall systems over gum arabic. The use of relatively low wall solids concentration would allow preparing emulsions of relatively low viscosity. High viscosity has adversely affected retention of volatiles during microencapsulation by spray drying due to difficulties associated with the atomization stage which resulted in droplets with low surface to volume ratio (Rosenberg, 1985).

Usually, a wall-to-core ratio of 4:1 (emulsion stage) is used in volatile microencapsulation by spray drying because of low volatile retention at higher volatile loads (Reineccius, 1988). High ester retention in our WPI/COH based wall systems could be achieved at 30% ester load. Using the highest possible core-to-wall ratio that provides high volatile retention is advantageous because the effect of wall materials (after reconstitution of capsules) on physicochemical characteristics of the product could be minimized.

Microstructure

The outer topography of the microcapsules was affected by wall composition (Fig. 2). Wall systems containing high proportions of carbohydrates, and especially MD5 and MD10, were associated with a very high extent of surface indentations. The limited surface indentations observed with increasing proportions of WPI indicate the importance of whey proteins in influencing the drying properties and the rheological characteristics (at very high concentrations) of the wall systems (Rosenberg and Young, 1993; Young et al., 1993b). Additionally, the observed structural differences may indicate different effective surface-to-volume ratios during drying of the different systems we hypothesized that these differences also affected the ECY retention during drying and may thus account, to some extent, for the various retention levels obtained with different wall systems.

The structural analyses revealed surface cracks in systems containing high proportions of MD5 or MD10 (Fig. 2) and their absence in those containing MD15 or CSS. This was in agreement with reported microstructural characteristics of maltodextrin-based microcapsules and indicates the brittle nature of the dry matrices in those samples (Rosenberg, 1985, Sankarikutty et al., 1988). The absence of surface cracks in systems containing high DE carbohydrates indicated that the film-forming properties were better than those with low DE maltodextrins. This confirmed reports of Anandaraman and Reineccius (1986) regarding good protection against core oxidation provided by high DE maltodextrins.

Table 2—Retention (%) of ethyl caprylate in wall systems consisting of mixtures of whey protein isolate (WPI) with various carbohydrates (Wall solids 25% (w/w) and ester load 30% (w/w of wall solids))

	Ratio of WPI to carbohydrates			
	1:19	*1:9*	*1:1*	*3:1*
Maltodextrin (DE = 5)	70.3^{d}	75.4^{c}	82.3^{abx}	84.7^{ax}
Maltodextrin (DE = 10)	86.9^{abx}	91.4^{ax}	73.9^{c}	81.6^{bx}
Maltodextrin (DE = 15)	79.7^{b}	90.1^{ax}	80.0^{bx}	79.2^{bx}
Corn syrup solids (DE = 24)	85.5^{abx}	90.7^{ax}	81.0^{bx}	90.4^{ax}

[a-c] Means within same row followed by different letters significantly different ($P < 0.05$).

[x] Means within same column not significantly different ($P < 0.05$).

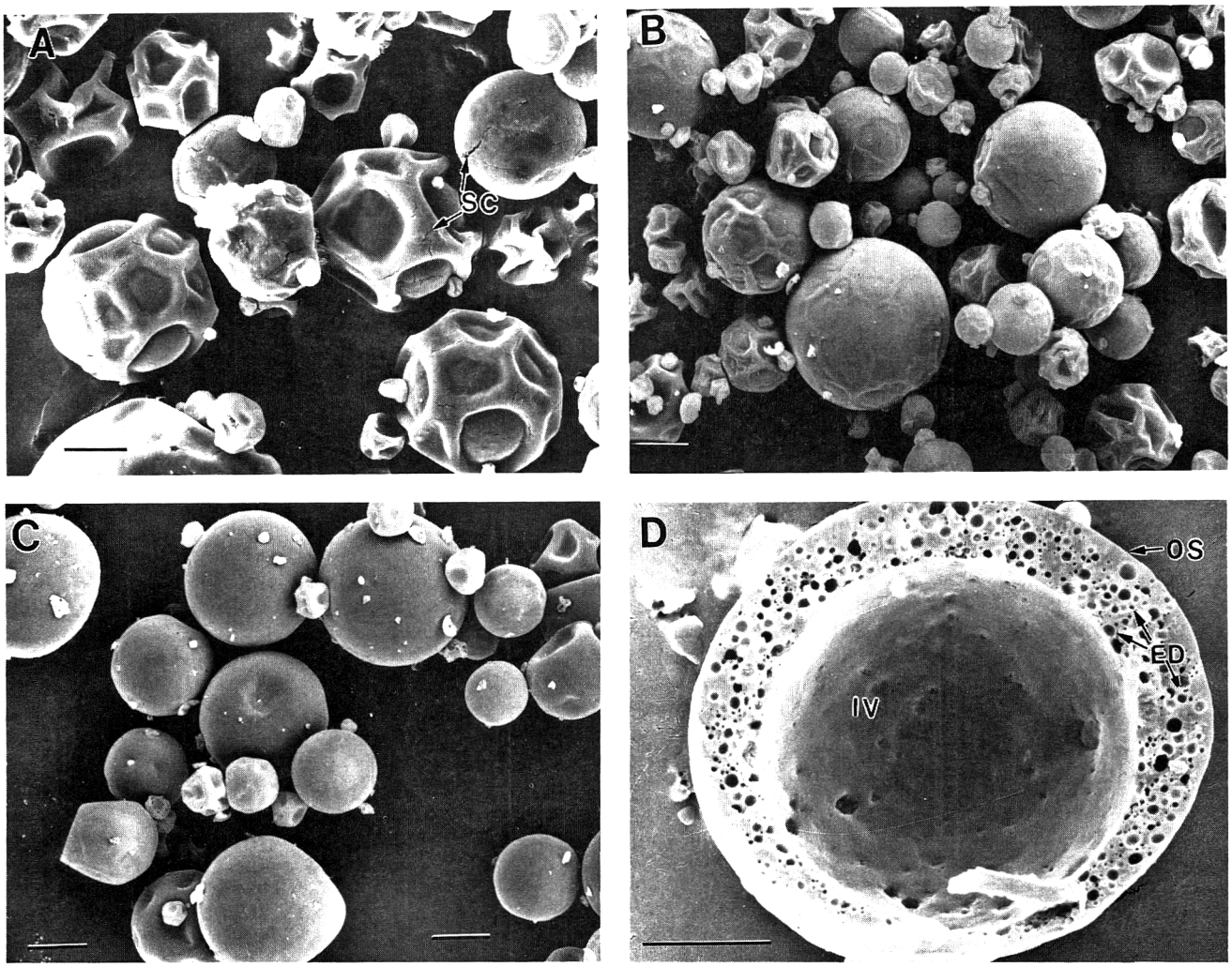


Fig. 2—Representative micrographs showing the outer topography (A-C) and inner structure (D) of ECY-containing microcapsules. Wall composition: (A) WPI/MD5 (1:19); (B) WPI/MD15 (1:19); (C) WPI/MD15 (3:1); (D) WPI/CSS (1:9). ED-ester droplets, IV- inner void, OS- outer surface, SC- surface cracks. Total solids of continuous phase 25% (w/w) and ester load 30% (w/w of wall solids). Bar = 10 μm for A to C, and 5 μm for D.

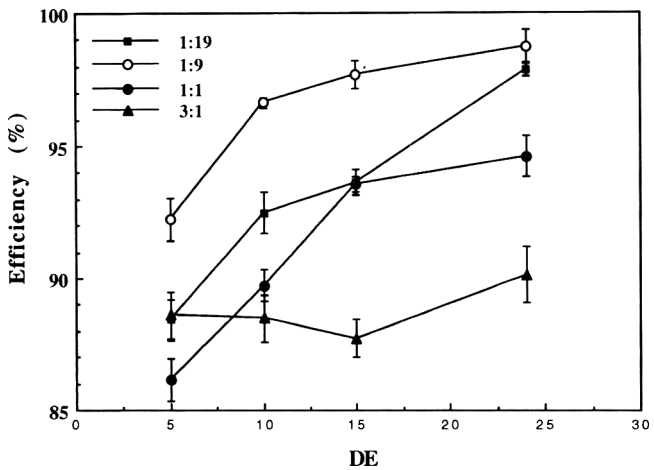


Fig. 3—Proportion of ECY that was not extracted by petroleum ether from microcapsules as related to carbohydrate DE value at different WPI/COH ratios. Total solids of continuous phase 25% (w/w) and ester load 30% (w/w of wall solids).

The inner structure of the capsules (Fig. 2D) indicated that in all cases the core material was in the form of small droplets embedded in the wall matrix. The inner structure of the capsules was similar to that reported for spray-dried, volatiles containing microcapsules in general, and to that of whey protein-based ones as well (Rosenberg et al., 1985, 1988; Rosenberg and Young, 1993; Rosenberg and Sheu, 1994). In all samples, a central void, and in some cases several connected voids characterized the spray-dried microcapsules. The formation of central voids in spray-dried particles has been reported and discussed. It was attributed to expansion (or "ballooning") associated with temperature increase within the drying particle during late stages of drying (Rosenberg et al., 1985, 1988). Thus, wall systems consisting of WPI and low DE maltodextrins at a WPI/COH ratio of at least 1:1, and those consisting of WPI and high DE maltodextrins or CSS provided structural features effective for microencapsulation.

Core extractability

The proportions of ester that could not be extracted from the dry capsules (EE) were compared (Fig. 3). Results indicated that EE was affected by type of carbohydrate and by its proportion in the wall. Increasing the DE value of the carbohydrate limited the proportion of ester that could be extracted. The highest EE values were obtained at a WPI/COH ratio of 1:9. The amount of core material that can be extracted from whey protein-containing, spray-dried microcapsules was demonstrated to represent the proportion of core that was on the surface of the dry particles and a proportion of truly encapsulated core that was extracted by leaching (Young et al., 1993a, 1993b; Moreau and

Rosenberg, 1993; Sheu and Rosenberg, 1993). Those reports suggested that carbohydrates limited core extractability by increasing the hydrophilic nature of the WPI/COH wall system (Young et al., 1993b). In the case of lactose, increasing EE was associated with formation of a glass phase consisting of amorphous lactose (Moreau and Rosenberg, 1993; Young et al., 1993a). The positive effect of DE value on EE could be attributed to increasing proportions of low molecular weight carbohydrates with increasing DE. Overall, lowest EE values were obtained with wall systems having a WPI/COH ratio of 3:1, almost regardless of DE value. In most cases, EE values for systems containing carbohydrates with DE >5 were higher than those of WPI-based, ECY-containing microcapsules (87%) prepared under the same conditions. Thus, we expected that a higher proportion of COH in the wall system would result in high EE values. However, the systems containing high proportions of MD5 or MD10 resulted in relatively low EE. This could be attributed to the effects of surface cracks identified on the microcapsules (Fig. 2). Additionally, the proportion of unencapsulated (or surface) core has been shown to be inversely proportional to the mean particle size of the core in the emulsion (Risch and Reineccius, 1988). The relatively low EE values for systems containing MD5 or MD10 at a WPI/COH ratio of 1:19 or 1:9 could be attributed to the relatively coarse nature of the emulsions (Fig. 1 and Table 1) that probably resulted in higher proportions of surface core than in other emulsions.

CONCLUSIONS

WALL SYSTEMS consisting of WPI and high DE maltodextrins or CSS are effective for volatile microencapsulation by spray drying. Such wall systems provide high volatile retention levels and limit the proportion of solvent extractable core. Low DE maltodextrins when incorporated at a high proportion into WPI-containing wall systems adversely affected the emulsion characteristics and caused structural defects in the dry product.

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Whey Protein Antigenicity Reduction by Fungal Proteinases and a Pepsin/Pancreatin Combination

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ABSTRACT

Whey protein components were hydrolyzed with Corolase 7092™ (peptidases from *Aspergillus* strains), pepsin and Corolase PP™ (a mixture of pancreatic enzymes), either individually or in combination, in trials to eliminate protein allergenicity. The hydrolysates were characterized by physico-chemical and by immunological techniques using sera from patients allergic to milk proteins. Enzyme specificity rather than degree of hydrolysis or molecular mass distribution of hydrolysates determined the residual antigenicity of the whey proteins. Ultrafiltration was a prerequisite for obtaining hypoallergenic whey protein hydrolysates.

Key Words: antigenicity, whey protein, fungal proteinases, pepsin, pancreatin

INTRODUCTION

INFANTS FED FORMULAS based on cow milk proteins may exhibit allergic symptoms to the proteins (Taylor, 1986; Wilson and Hamburger, 1988), possibly due to enhanced intestinal mucosal permeability. Many adverse reactions are mediated by immunoglobulin E (IgE) antibodies produced against these proteins, which in turn lead to release by mast cells of inflammatory mediators such as histamine and serotonin. β-Lactoglobulin (βLg) is considered the most important allergen because it is the main protein of cow milk which is not present in human milk (Bell and McKenzie, 1964; Taylor, 1986). In addition, it appears to resist enzymatic digestion by pepsin in the stomach (Yvon et al., 1984; Scanff et al., 1990) and may, therefore, pass undigested through the gut epithelial cells into blood circulation. Indeed, by use of anti-bovine βLg antibodies, low concentrations of βLg have been detected in human milk (Mäkinen-Kiljunen and Palosuo, 1992; Lovegrove et al., 1993). However, sensitivity to other cow milk proteins has also been reported (Goldman et al. 1963; Kuitunen et al., 1975; Lebenthal, 1975) and specific IgE antibodies against all major milk proteins have been found in sera of allergic persons (Gjesing et al., 1986; Otani et al., 1989a, b).

The most common means to reduce allergenicity of cow milk is enzymatic hydrolysis of its principal proteins, namely, βLg, α-lactalbumin (αLa), bovine serum albumin (BSA), bovine serum immunoglobulin G (B-IgG) and the caseins. The choice of the proteolytic enzyme for in vitro hydrolysis is important. The enzyme's specificity influences both the degradation of the epitopes responsible for immunological reactions and the formation of bitter peptides. For reduction of whey protein antigenicity, proteinases involved in human digestion have often been used to manufacture hydrolysates. Many studies (Haddad et al., 1979; Schwartz et al., 1980; Jost et al., 1987; Asselin et al., 1988, 1989; Fritsché and Bonzon, 1990) have proved the presence in the blood system of IgE antibodies to digests of αLa and/or βLg as well as to digests of BSA and B-IgG (Jost et al., 1987). Remaining antigenic determinants were also found after hydrolysis with other enzyme systems such as plant proteinases (papain, bromelain) or combinations of these with bacterial proteinases (Nakamura et al. 1993a,b). Subsequent ultrafiltration of hydrolysate appeared to be a prerequisite to eliminate antigenicity of hydrolyzed whey protein (Pahud et al., 1985). Poulsen and Hau (1987) and van Beresteijn et al. (1994) have shown that peptides from whey protein with apparent molecular mass below 3400 Da did not elicit an IgE-mediated allergic reaction.

Several hypersensitivity reactions have been reported for infant formulas containing hydrolyzed whey protein (Businco et al., 1989; Heyman et al., 1990; Ellis et al., 1991; Businco et al., 1994). These could be ascribed to the presence of epitopes to which 25% of those infants who suffer from cow milk allergy have been sensitized (Collins-Williams, 1992). IgE binding to components of commercially available whey protein hydrolysates has been shown in sera from children allergic to cow milk (Plebani et al., 1990; Oldaeus et al., 1991; Rugo et al., 1992). Those findings indicated the need to improve existing formulas, possibly by exploring new enzyme systems.

Our objective was to investigate the degradation of the major individual whey allergens, (αLa, βLg and BSA) by a food-grade enzyme preparation of fungal proteinases (Corolase 7092™) and a combination of pepsin and pancreatic enzymes at different stages of the enzyme reaction. We chose Corolase 7092 because, according to the manufacturer's claims (Röhm, 1989), it combined high proteolytic activity with a low tendency to form bitter peptides. The loss of protein antigenicity was determined by means of binding studies with cow milk protein-specific human-IgE (H-IgE) and IgG (H-IgG) using a sensitive competitive enzyme-linked immunosorbent assay (ELISA). The molecular characteristics of hydrolysates were determined by the average degree of hydrolysis (DH), electrophoresis in sodium dodecyl sulfate-containing polyacrylamide gels (SDS-PAGE) and gel permeation chromatography (GPC).

MATERIALS & METHODS

Patient serum samples

Serum samples from children and adults with clinically allergic symptoms to cow milk were donated by the Departments of Pediatrics of the Academic Hospital of the Free University, Amsterdam, and the Wilhelmina Children's Hospital, Utrecht. Sera were tested for specific IgE and IgG binding to individual milk proteins by ELISA (see below). Three sera from allergic patients (AS) were selected: AS I, an adult serum with high IgE antibody titer to αLa, βLg A and B and whole casein; AS II, an adult serum with high IgG antibody titer to these proteins, and AS III, consisting of pooled sera of 5 children with high IgE and IgG antibody titers to BSA and B-IgG. A normal control serum (NCS), consisting of the pooled sera of 8 non-allergic persons to milk proteins, was a control.

Materials

Whey protein concentrate (WPC), a spray-dried product, was manufactured at our Institute by ultrafiltration of cheese whey; it contained 86% protein. The proteins αLa and βLg A and B were prepared from cheese whey by chromatography (Schmidt and van Markwijk, 1993). BSA (A 4378) and B-IgG (I 5506) were purchased from Sigma Chemical Company (St. Louis, MO). Whole casein was prepared by isoelectric precipitation from skim milk with HCl at pH 4.6. The precipitated casein

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was thoroughly washed with distilled water, redissolved by addition of NaOH to pH 7.0 and then lyophilized.

Corolase 7092, a liquid technical food-grade enzyme mixture of endopeptidases and exopeptidases from *Aspergillus* strains, and Corolase PP™, a mixture of enzymes from porcine pancreas, were purchased from Röhm (Darmstadt, Germany). Porcine pepsin (P 7000) came from Sigma. The activities of the various enzymes were 2000 proteinase units/mg for Corolase 7092 (substrate hemoglobin), 350 proteinase units/mg for Corolase PP (substrate casein) and 550 proteinase units/mg for pepsin (substrate hemoglobin). For definition of proteinase units and enzyme activities see product information sheets (Röhm, 1988a, b).

Enzymatic hydrolysis of whey proteins

Protein hydrolysates were produced from an aqueous solution of WPC (5% protein) or from 0.67% solutions of individual whey proteins by the pH-stat technique (Adler-Nissen, 1982). Since in preliminary experiments (data not shown) we had not found differences in antigenic behavior between variants A and B of βLg, we only used βLg A in further experiments. The following hydrolysis schemes were used:

A. Protein solutions were incubated with Corolase 7092 at 40°C and pH 7.5. To obtain a gradual increase in DH we used 10 μL enzyme solution/g protein for 1 hr, followed by 2 hr with 100 μL enzyme solution/g protein. At predetermined times (15 to 180 min), aliquots of the digest were taken and incubated another 15 min at 60°C to allow further hydrolysis. This second incubation was done because we had found that the optimum temperatures for hydrolysis of βLg and αLa by Corolase 7092 were 40 and 60°C, respectively. Enzyme inactivation was by means of heating the samples for 5 min at 95°C.

B. In order to compare enzymatic modification by Corolase 7092 with physiological digestion, protein solutions were incubated with pepsin (20 mg/g protein) at pH 2.0 for 100 min at 37°C. Pepsin was inactivated by increasing the pH to 7.5. Thereafter, Corolase PP (30 mg/g protein) was added and hydrolysis was continued for 4 hr at 37°C. To inactivate Corolase PP samples were heated for 5 min at 95°C.

C. To simulate physiological digestion of a Corolase 7092 WPC-digest, WPC was first hydrolyzed with Corolase 7092 (100 μL enzyme solution/g protein) for 3 hr at 40°C. At predetermined times aliquots of the digest were removed and incubated another 15 min at 60°C to allow further hydrolysis. Thereafter, the enzyme in the aliquots was inactivated by heating the samples for 5 min at 95°C. This 180 min WPC digest was further hydrolyzed with pepsin/Corolase PP according to scheme B.

In order to determine effects of temperature alone on reduction of antigenicity through protein denaturation, the whey proteins were successively heated for 15 min at 60°C and 5 min at 95°C without enzyme.

After heating, all samples were cooled in ice water and sodium azide was added (0.02%) for preservation. Aliquots of samples with longest hydrolysis times were subjected to ultrafiltration through a 3000 Da membrane (YM3, Amicon, Danvers, MA). All collected samples were stored at −20°C until further analysis. For in vitro antigenic properties, however, the hydrolysates were used without previous freezing to prevent formation of protein aggregates.

Chemical characterization of hydrolysates

Degree of hydrolysis (DH). The average DH reached in each sample was calculated from the increase in α-amino nitrogen, determined by the *o*-phthaldialdehyde (OPA) method (Church et al., 1985). Following Frister el al. (1988), we used n,n-dimethyl-2-mercaptoethyl-ammonium chloride instead of 2-mercaptoethanol (van Beresteijn et al., 1994).

Electrophoresis and determination of the molecular mass distribution. The hydrolysates were subjected to SDS-PAGE using the Phast system and high-density gels (Pharmacia, Uppsala, Sweden). The total protein concentration in the samples was ≈0.2%. Two standard protein mixtures, Bio-Rad LMW standard (Bio-Rad, Richmond, CA) and BDH 44247 (BDH Laboratory Supplies, Poole, Dorset, UK), were used as molecular mass markers. After electrophoresis the proteins and peptides were stained with silver as described in the Phast Gel™ Silver Kit Instruction Manual. This method was 100 times more sensitive than Coomassie blue staining. Molecular mass distributions of peptides in the hydrolysates were determined by GPC on a Superdex-75 HR 10/30 column (Pharmacia/LKB, Uppsala, Sweden). Elution was at room temperature (≈23°C) with a 0.125M potassium phosphate - 0.125M sodium sulfate buffer of pH 6.65. Peptide concentrations in the eluate were determined from absorption at 220 nm. Calculations were made on the assumption of an exponential relationship between molecular mass (M_r) and elution time, and taking into account apparent M_r-regions based on calibration with a series of reference compounds (Visser et al., 1992).

Immunological properties of hydrolysates

We screened patient serum samples for binding capacities of H-IgE and H-IgG antibodies to WPC and individual milk proteins. We also tested the residual antigenicity of the milk protein hydrolysates. We used the ELISA method as described by Fritsché and Bonzon (1990) with slight modifications.

Indirect ELISA. Polystyrene microtiter plates (655061, Greiner, Alphen a/d Rijn, The Netherlands) were used as solid support. Single wells were coated with 135 μL/well of antigen (individual milk proteins, 37 μg/mL in 0.05 M sodium bicarbonate buffer, pH 9.6) and incubated overnight at 4°C. Residual free binding sites were blocked with 200 μL/well of 0.4% fish gelatin (G 7765, Sigma) in phosphate-buffered saline (0.15M NaCl, 0.01M phosphate buffer, pH 7.4) containing 0.05% Tween 20 (P 1379, Sigma) (PBS-Tween) for 1 hr at room temperature. Plates were incubated with 100 μL/well of AS diluted in PBS-Tween (1:25 for IgE and 1:1000 for IgG binding) for 2 hr at room temperature. The same dilutions of NCS were used as negative controls. The H-IgE and H-IgG antibodies which reacted with plate-bound antigens were determined by use of either peroxidase-conjugated goat anti-H-IgE (ε-chain) or peroxidase-conjugated goat anti-H-IgG (H and L chains) (Nordic Immunological Laboratories, Tilburg, The Netherlands). These were diluted 5000-fold in PBS-Tween containing 0.4% fish gelatin and were incubated 1 hr at room temperature (≈23°C). As enzyme substrate a solution of freshly prepared o-phenylene-diamine dihydrochloride (P 1526, Sigma) 1 mg/mL in 0.05M citrate, 0.1 M potassium phosphate buffer, pH 5.0, containing 0.012% hydrogen peroxide, was used. After incubating plates for 30 min at room temperature, 100 μL of 2N sulfuric acid was added. Optical densities were read at 490 nm on an automated ELISA plate reader (Dynatech MR 7000, Trade Tek, Rotterdam, The Netherlands). ELISA determinations were carried out in duplicate and measurements averaged.

Inhibition ELISA. The relative antigenicity of hydrolysates was evaluated by measuring the ability of the hydrolysates to inhibit binding of specific H-IgE or H-IgG antibodies to ELISA plates coated with αLa, βLg, BSA, B-IgG or casein. Single wells of ELISA plates were coated with 135 μL of αLa, βLg, BSA, B-IgG or casein (15 μg/mL in 0.05M sodium bicarbonate buffer, pH 9.6). In 1.5-mL glass vials, serial 9-fold dilutions of the hydrolysate samples in PBS-Tween were incubated overnight at 4°C with a constant amount of AS (1.5% for IgE or 0.05% for IgG binding). Residual unbound H-IgE and H-IgG antibodies were determined by the ELISA method described above by applying 100 μL of preincubated AS to each well of the coated ELISA plates. The percentage of inhibition was calculated in relation to the maximum inhibition (vials containing NCS instead of AS) and the minimum inhibition (vials without hydrolysate). For this purpose an inhibitor concentration of 1 g/L was used. It was the highest concentration at which differences in inhibition for the various hydrolysates were still clear and it was also the lowest concentration which produced an almost 100% inhibition for all individual milk proteins tested (Fig. 1).

Reliability of results

Sera from patients allergic to cow milk with high concentrations of antibodies against the different individual milk proteins are scarce and difficult to obtain. Most patients are infants from whom only occasionally small amounts of serum can be obtained. The amounts of serum available were not sufficient for duplicate experiments. We determined the precision of methods used for characterization of hydrolysates. We performed additional hydrolyses of WPC with pepsin and Corolase PP (scheme B). The repeatability standard deviation (S_r) according to ISO 5752-1986 (International Organization for Standardization, 1989) of the physico-chemical and immunological assays was estimated from duplicate measurements. For immunological assays we used patient serum AS II. S_r for DH determination was 0.99% (degrees of freedom (d.f.) = 48), for the molecular mass distribution 0.29% (d.f. = 40) and for the ELISA, including between well and between plate variation, 1.77% (d.f. = 19).

RESULTS & DISCUSSION

Chemical characterization of hydrolysates

Individual whey proteins and WPC were hydrolyzed with Corolase 7092, pepsin and Corolase PP either individually or in combination. Since the various proteolytic enzymes had different specificities, they hydrolyzed the whey proteins differently, as clearly demonstrated by gel electrophoretic patterns (Fig. 2). The protein samples which were heated in absence of enzyme

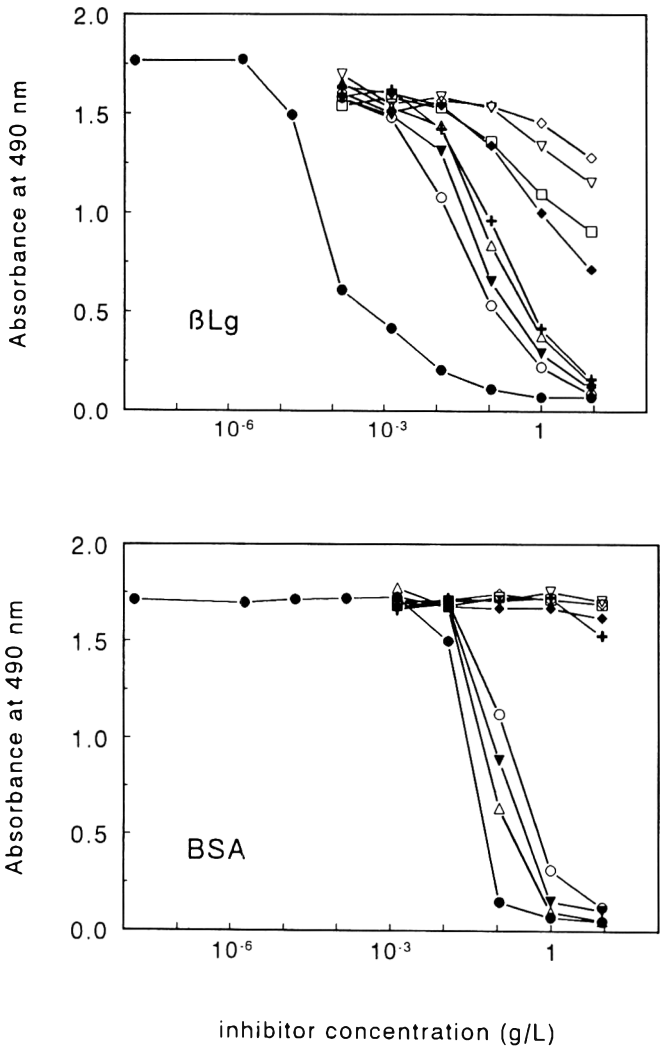


Fig. 1—Inhibition of human IgE-binding to βLg and BSA by WPC (●) and by WPC after hydrolysis according to enzyme system C: Corolase 7092 for 10 min (○), 60 min (▼), 180 min (△), subsequent hydrolysis with pepsin for 90 min (+), and Corolase PP for 10 min (◆) and 180 min (□). (▽) 3000 Da permeate of the 180 min Corolase 7092 hydrolysate, (◇) 3000 Da permeate of the final WPC hydrolysate. Patient serum (AS) I was used to determine residual antigenicity against βLg and AS III against BSA. Absorption values are from duplicate measurements.

exhibited electrophoretic patterns which were indistinguishable from those of unheated samples (data not shown). Changes in molecular mass distributions of the hydrolysates parallelled the results of the gel electrophoresis (data not shown). The final DH of the hydrolysates was of the same magnitude for Corolase 7092 (hydrolysis scheme A) and for the combination of pepsin and Corolase PP (hydrolysis scheme B) (Tables 1 and 2). However, when WPC, which had been predigested with Corolase 7092, was further hydrolyzed with pepsin and Corolase PP (hydrolysis scheme C), the DH increased from 37.9% to 54.3% (Table 2). This emphasized the different specificities of the two enzyme systems. We found that the α-amino nitrogen content of the samples did not significantly increase during heating without enzyme which indicated that heating alone did not lead to hydrolysis.

Individual whey proteins. βLg was completely broken down (Fig. 2) by Corolase 7092 to peptides which were no longer detected by electrophoresis, whereas in the hydrolysates of αLa and BSA large peptides remained. The combination of pepsin and Corolase PP appeared to be more effective in eliminating large peptides although that pepsin hydrolysed βLg not very

Table 1—Degree of hydrolysis (DH) and molecular mass distribution of hydrolysates of individual whey proteins, hydrolysis schemes and times[a]

Hydrolysis scheme A: COROLASE 7092[b]

Protein	DH (%)	Molecular mass distribution (%) >10kDa	10kDa-5kDa	5kDa-3kDa	<3kDa
αLa	37.7	2.6	20.0	19.5	57.9
βLg	35.8	2.0	26.3	23.4	48.3
BSA	30.2	10.4	20.9	20.5	48.1

Hydrolysis-scheme B: PEPSIN/COROLASE PP

Protein	Enzyme	DH (%)	Molecular mass distribution (%) >10kDa	10kDa-5kDa	5kDa-3kDa	<3kDa
αLa	Pepsin[c]	2.4	18.9	29.6	27.9	23.6
	Cor. PP[d]	33.6	0.1	5.0	22.0	79.9
βLg	Pepsin[c]	2.4	70.4	11.9	7.2	10.5
	Cor. PP[d]	33.6	2.6	19.0	30.6	47.8
BSA	Pepsin[c]	6.8	40.3	18.8	8.5	32.8
	Cor. PP[d]	35.6	2.8	18.9	24.0	54.4

[a] Values are from single hydrolysis experiments.
[b] Corolase 7092 for 180 min.
[c] Pepsin for 100 min.
[d] Pepsin (100 min) followed by Corolase PP for 210 min, total incubation time 310 min.

Table 2—Degree of hydrolysis (DH) and molecular mass distribution of WPC hydrolysates from hydrolysis schemes at different times[a]

Hydrolysis scheme B: PEPSIN/COROLASE PP

Hydrolysis time (min)	DH (%)	Molecular mass distribution (%) >10kDa	10kDa-5kDa	5kDa-3kDa	<3kDa
Pepsin					
0	—	99.3	0.6	0.1	0.0
100	2.1	61.9	15.5	10.6	12.0
Corolase PP					
130	16.0	41.9	12.8	16.6	28.7
190	23.9	15.6	15.5	23.3	45.5
310	29.1	5.8	13.2	25.7	55.3
310 (3000 Da)[b]	—	0.4	7.6	25.6	66.4

Hydrolysis scheme C: COROLASE 7092 and PEPSIN/COROLASE PP

Hydrolysis time (min)	DH (%)	Molecular mass distribution (%) >10kDa	10kDa-5kDa	5kDa-3kDa	<3kDa
Corolase 7092					
0	—	97.6	0.7	0.3	1.3
10	19.6	13.7	24.4	22.5	39.3
60	25.6	11.1	19.8	25.2	44.0
180	37.9	11.2	13.2	24.2	51.4
180 (3000 Da)[b]	—	0.3	11.8	27.3	60.6
Pepsin					
270	39.3	2.4	14.4	28.9	54.3
Corolase PP					
280	43.4	5.4	11.7	27.1	55.9
300	47.6	4.7	10.7	26.3	58.4
330	51.9	4.3	9.3	26.2	60.2
450	54.3	3.3	7.6	25.2	63.9
450 (3000 Da)[b]	—	0.0	4.4	25.1	70.5

[a] Values are from single hydrolysis experiments.
[b] Hydrolysate after ultrafiltration through a filter with a molecular mass cut-off value of 3000 Da.

thoroughly. Results shown correlated well with molecular mass distributions (Table 1).

Note differences in proteolysis of αLa and βLg by pepsin. With both proteins a DH of 2.4% was achieved after 100 min incubation (Table 1). During this incubation αLa was completely broken down to peptides with molecular masses around 6000 Da, whereas under the same conditions a large part of βLg remained almost intact (Fig. 2 and Table 1). From these results we concluded that in αLa a few peptide bonds were hydrolyzed

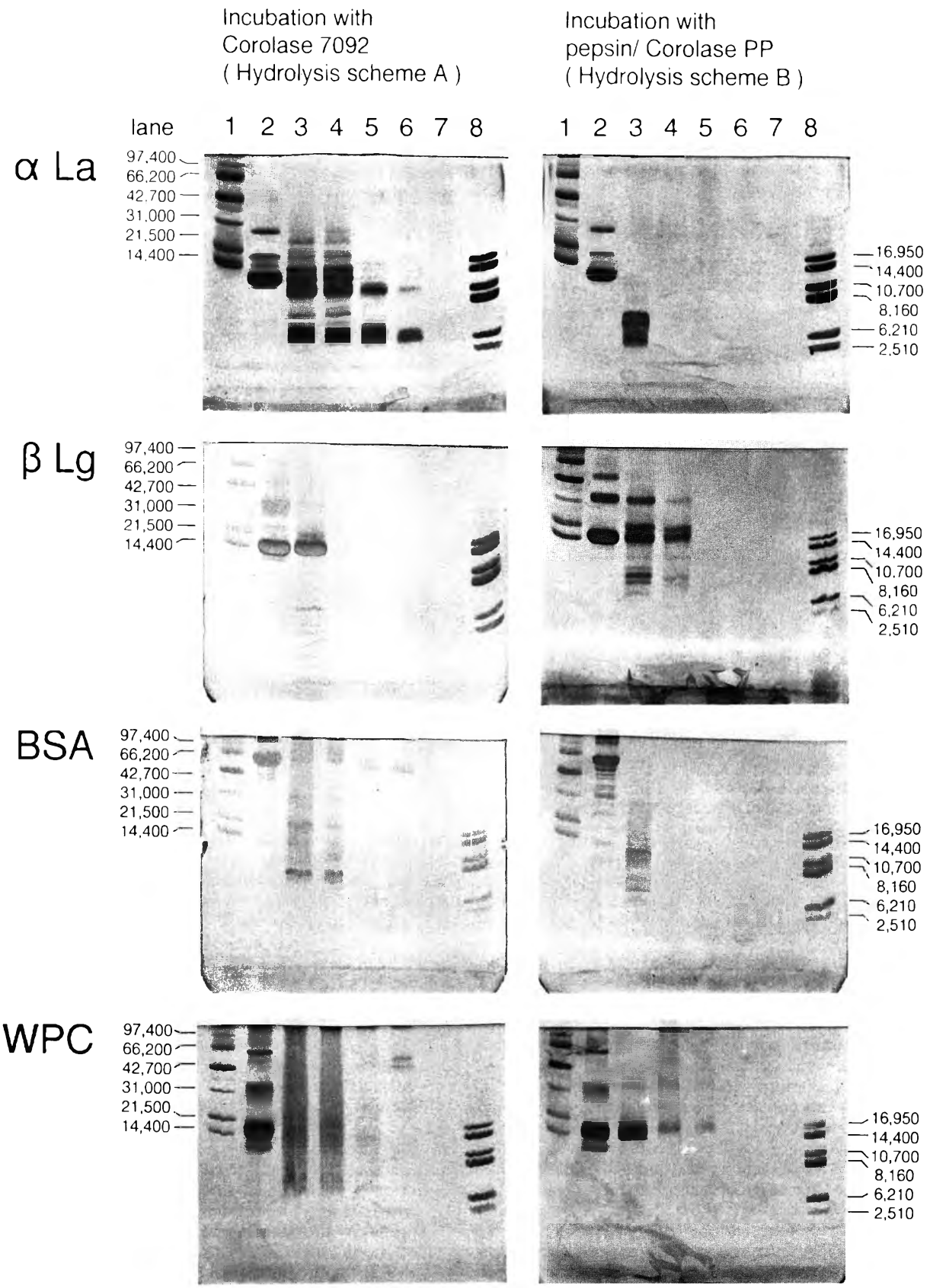


Fig. 2—SDS-PAGE patterns of hydrolysates of individual whey proteins, obtained by hydrolysis schemes A (Corolase 7092) and B (pepsin/Corolase PP). In all gels, lanes 1 and 8 contain reference samples (Bio-Rad LMW standard and BDH 44247, respectively). Molecular masses in the reference samples are indicated along the gels. In gels of hydrolysis scheme A, times of hydrolysis, t, are in lanes 2, 0; lanes 3 15 min; lanes 4 60 min; lanes 5 75 min; lanes 6 180 min; lanes 7 contain 3000 Da permeate of the 180 min hydrolysate. For αLa, βLg and BSA the enzyme concentration was increased 10 times after 60 min. In gels of hydrolysis scheme B the times of hydrolysis are in lanes 2, 0; lanes 3, 100 min; lanes 4, 130 min; lanes 5, 190 min; lanes 6, 310 min; lanes 7 contain 3000 Da permeate of the 310 min hydrolysate.

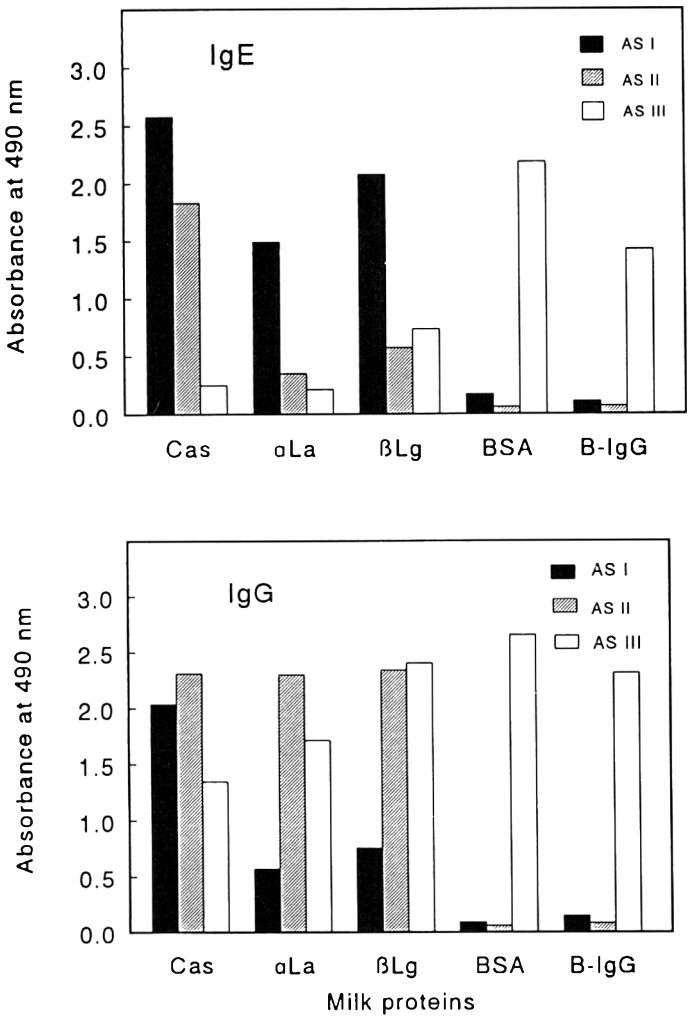


Fig. 3—Specific IgE and IgG binding of casein (Cas), αLa, βLg, BSA and B-IgG of patient sera AS I, AS II and AS III. Protein concentration of coating antigen was 75 μg/mL; sera were 25-fold diluted for IgE and 1000-fold for IgG.

in the middle of the molecule, and that in βLg one or more small peptides were split from the terminal site of the polypeptide chain.

B-IgG had to be excluded from our comparative studies because its hydrolysis with Corolase 7092 according to scheme A could not be performed. Precipitation of the protein occurred during inactivation of the enzyme (5 min, 95°C) even after 3 hr hydrolysis. This was undoubtedly due to insufficient hydrolysis of the B-IgG by Corolase 7092 since the alkali uptake at the pH-stat was minimal during incubation with the enzyme.

Whey protein concentrate. In general the results of the hydrolysis of WPC by the enzymes agreed with those with individual whey proteins (Fig. 2). The degradation of αLa and βLg in WPC by Corolase 7092, however, appeared to differ somewhat from that of the isolated proteins. For a valid comparison, however, note that in the WPC experiment enzyme concentration was 100 μL/g protein from the beginning, whereas in the other experiments hydrolysis was started with only 10 μL/g protein during the first hour. Furthermore, the concentration of αLa in WPC was comparatively low and, consequently, certain peptide fractions obtained after hydrolysis may have been undetectable in the gel. Also, the WPC we used had been spray-dried, while the isolated αLa and βLg samples had been freeze-dried. Therefore, some heat denaturation may have occurred in the WPC, which could have affected the hydrolysis reaction (Schmidt and van Markwijk, 1993). In the hydrolysate of WPC (by scheme C) a small amount of high-molecular-mass material, originating from BSA, was present after incubation with Corolase 7092. This agreed with results with BSA alone (Tables 1 and 2). Further hydrolysis with pepsin and Corolase PP resulted in peptides which could no longer be detected by gel electrophoresis (gel not shown).

Even at high DH values (> 30%, corresponding to average chain length of 3-4 amino acids), none of the enzyme systems completely removed all high-molecular-mass material. The manufacture of protein hydrolysates for use in hypoallergenic formulas, in which large peptides are undesirable, therefore requires an ultrafiltration step following hydrolysis. A filter with a molecular mass cut-off of 3000 Da removed most of the remaining high-molecular-mass material in final hydrolysates of the three incubation schemes (Fig. 2, lanes 7 and Table 2).

In spite of similar final DH values the combination of pepsin and Corolase PP was more effective in destroying the principal whey protein allergens than was Corolase 7092. This was undoubtedly connected with the differences in specificity. Corolase 7092 is a mixture of endo- and exopeptidases. The endopeptidases have not been characterized with respect to cleavage specificity but the exopeptidases preferentially split off amino acids with hydrophobic side chains (notably leucine, alanine and glycine) from the N-terminus of peptides (Röhm, 1989). A high DH value may thus be a result of many free amino acids, whereas the number of hydrolyzed peptide bonds elsewhere in the peptide chain would be low, leaving a relatively large number of antigenic epitopes intact. Pepsin and Corolase PP, the latter consisting mainly of trypsin and some chymotrypsin, barely showed exopeptidase activity. Hydrolysis with these enzymes occurs throughout the peptide chain, pepsin splitting preferentially adjacent to amino acids with hydrophobic aromatic side chains (phenylalanine and tryosine) trypsin adjacent to arginine and lysine, and chymotrypsin adjacent to amino acids with bulky hydrophobic side chains (e.g. tyrosine, tryptophan, phenylalanine and leucine, Webb, 1992). Simulated physiological digestion of a Corolase 7092 WPC hydrolysate, therefore, resulted in a further reduction of antigenic sites.

Antigenicity of hydrolysates

The specific IgE and IgG binding of three different patient sera, AS I, AS II and AS III to individual milk proteins was compared (Fig. 3). Because of its high specific IgE binding to αLa, βLg and casein, AS I was used to determine residual IgE antigenicity of these proteins in hydrolysates of WPC and individual milk proteins. For similar reasons, AS II was used to determine residual IgG antigenicity. Residual IgE and IgG antigenicity of BSA and B-IgG was determined by AS III. Residual casein antigenicity of WPC hydrolysates was tested because trace amounts of casein are always present in commercial whey preparations.

Binding inhibition, calculated at a protein inhibitor concentration of 1 g/L, of H-IgE and H-IgG antibodies against the individual milk proteins of the WPC hydrolysates was compared for increasing DH (Fig. 4, 5). Residual H-IgE and H-IgG antigenicity was clearly related to the specificities of the proteinases and differences occurred between the individual milk proteins. In general, the patterns agreed well with those obtained with hydrolysates of individual whey proteins. Those studies could be considered as a form of duplication and their corresponding findings were an indication of the reliability of the measurements. Heating the WPC solution successively 15 min at 60°C and 5 min at 95°C in the absence of enzyme had no significant effect on antigenicity of individual whey proteins. This agreed with results of Jost et al. (1987) who, however, reported a reduction of antigenicity by more severe heat treatments.

Whey protein concentrate, hydrolysis scheme B. In spite of the low DH (2.1%) the hydrolysis of WPC with pepsin at pH 2.0 completely eliminated the H-IgE antigenicity related to BSA (Fig. 4a). This could have possibly been caused by the low pH needed for the pepsin hydrolysis. To exclude that, we also in-

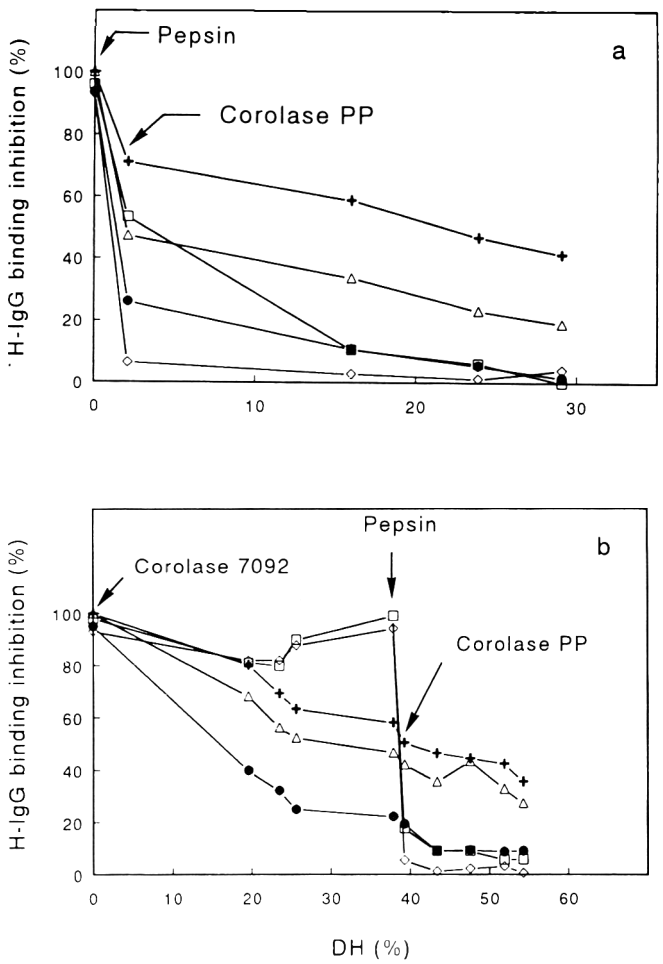


Fig. 4—Degree of hydrolysis and residual H-IgE antigenicity (percentage inhibition of H-IgE binding) vs individual milk proteins of whey protein hydrolysates achieved with (a) hydrolysis scheme B; (b) hydrolysis scheme C. Patient serum (AS) I used for determination of residual H-IgE antigenicity of αLa, βLg and casein, AS III for that of BSA and B-IgG. Concentration of protein inhibitors was 1 g/L. (+) αLa, (△) βLg, (●) casein, (◇) BSA and (□) B-IgG. Times at which different enzymes were added are indicated in the figures. Values from single hydrolysis experiments.

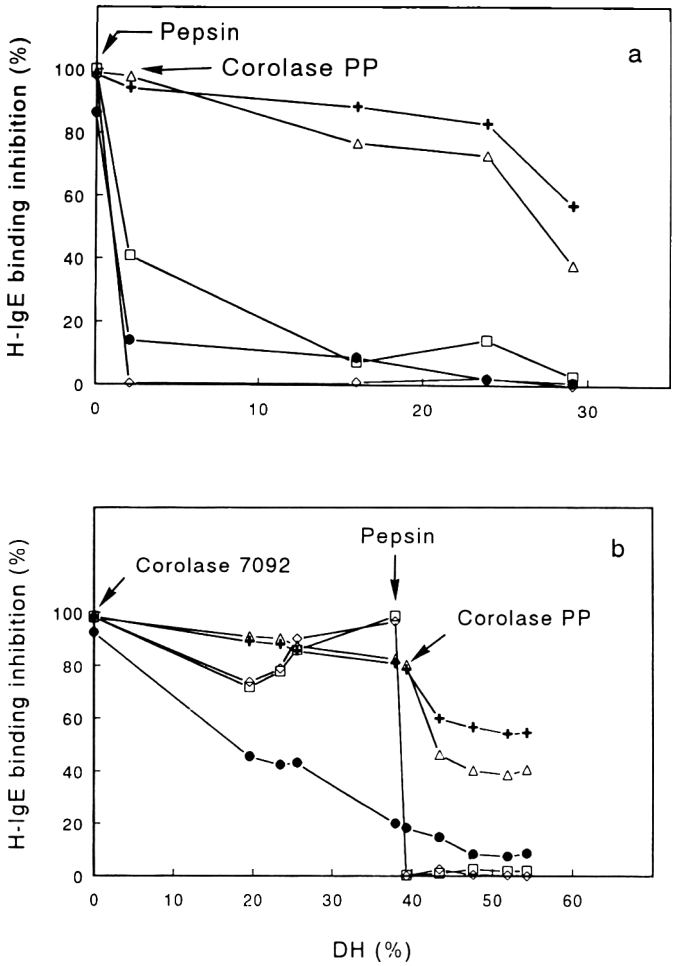


Fig. 5—Degree of hydrolysis and residual H-IgG antigenicity (percentage inhibition of H-IgG binding) vs individual milk proteins of whey protein hydrolysates achieved with (a) hydrolysis scheme B; (b) hydrolysis scheme C. Patient serum (AS) II used for determination of residual H-IgG antigenicity of αLa, βLg and casein, AS III for that of BSA and B-IgG. Concentration of protein inhibitors was 1 g/L. (+) αLa, (△) βLg, (●) casein, (◇) BSA and (□) B-IgG. Times at which different enzymes were added are indicated in the figures. Values are from single hydrolysis experiments.

cubated BSA for 100 min at pH 2.0 and 37°C without enzyme. A reduction in antigenicity was not observed. Apparently, specific cleavage sites for pepsin were involved in the breakdown of the IgE-binding epitopes of BSA. The pepsin treatment also strongly reduced the antigenicity of casein and B-IgG but had little effect on that of αLa and βLg. The residual antigenicity of casein and B-IgG completely disappeared after subsequent hydrolysis with Corolase PP, but some epitopes of αLa and βLg A remained intact.

Whey protein concentrate, hydrolysis scheme C. H-IgE antigenicity of αLa, βLg, BSA and B-IgG was hardly reduced after hydrolysis of WPC by Corolase 7092 (Fig. 4b). Antigenicity of casein was significantly reduced but not eliminated, even at high DH values. Surprisingly, antigenicity of BSA and B-IgG initially decreased (first 10-20 min of the hydrolysis) but increased with longer hydrolysis (Fig. 1, hydrolysis of BSA for 10 min (○), 60 min (▼) and 180 min (△) and, Fig. 4b). With DH-values of 30–40%, the antigenicity of the WPC hydrolysate for BSA and B-IgG was similar to that of native WPC. This increase in antigenicity was also seen in the BSA hydrolysates. A plausible explanation is that unfolding of the protein, resulting from the action of the enzyme, led to the exposure of more antigenic sites of the protein. The epitopes remaining intact, even after extensive hydrolysis, either did not contain specific cleavage sites for proteinases in Corolase 7092 or were not accessible to the enzyme in the native protein. Similar observations have previously been made for peptic and peptic-tryptic digests of αLa and βLg by Haddad et al. (1979) and by Asselin et al. (1989).

The remaining antigenic sites of BSA and B-IgG were completely destroyed during simulated physiological digestion by peptin and Corolase PP (Fig. 4b). The pretreatment facilitated breakdown of the epitopes of B-IgG by pepsin. In contrast, pretreatment slightly hampered the reduction of casein epitopes by Corolase PP. Epitopes of αLa and βLg remained intact and final residual antigenicities were similar whether WPC was pretreated with Corolase 7092 or not (Fig. 4a,b).

Ultrafiltration through a filter with molecular mass cut-off 3000 Da removed most remaining large peptides (Fig. 2 and Table 2). However, although molecular mass distribution profiles were similar, clear differences were found in residual antigenicity between the three WPC permeates (Table 3). Residual IgE antigenicity of BSA and B-IgG was absent in the three WPC permeates, but that of αLa and βLg was substantial. That of hydrolysis scheme B was higher than the others. Whether such residual antigenicity is of clinical significance requires further testing by in vivo methods. Van Beresteijn et al. (1994) reported that the 3000 Da permeate of a Corolase 7092 WPC hydrolysate did not elicit an IgE-mediated allergic reaction.

Table 3—Residual H-IgE antigenicity[a] vs individual milk proteins of WPC hydrolysates, from different hydrolysis schemes before and after ultrafiltration (cut-off 3000 Da)

	Inhibition (%)[b]					
	Scheme A[c]		Scheme B[d]		Scheme C[e]	
	Before	After	Before	After	Before	After
αLa	81.0	17.1	57.3	48.5	54.5	23.1
βLg	82.6	26.7	38.2	35.7	40.7	19.8
BSA	96.6	0.0	0.0	0.0	0.0	0.0
B-IgG	98.7	0.0	3.0	2.7	2.0	3.4
Casein	20.0	10.8	1.0	1.0	8.5	4.0

[a] Percentage of inhibition of H-IgE binding to the individual milk proteins by various whey hydrolysates. Values are from single hydrolysis experiments.

[b] Patient serum AS I was used for determination of residual IgE antigenicity of whey protein hydrolysates against αLa, βLg and casein, AS III against BSA and B-IgG. The concentration of the protein inhibitors was 1 g/L.

[c] Corolase 7092 for 180 min.

[d] Pepsin for 100 min followed by Corolase PP for 210 min, total incubation time 310 min.

[e] Corolase for 180 min followed by pepsin for 90 min and Corlase PP for 180 min, total incubation time 450 min.

The results of experiments on binding inhibition of H-IgG antibodies were similar to those for H-IgE antibodies, except that at the same DH, IgG-binding of αLa and βLg was less than IgE-binding. This resulted in a lower residual H-IgG antigenicity. Such difference was not observed for BSA, B-IgG and casein (Fig. 4 and 5).

Sera from cow milk allergic patients with a high concentration of antibodies against different milk proteins are difficult to obtain. Consequently, antisera from laboratory animals such as rabbit, rat and mouse have often been used to determine residual antigenicity of WPC hydrolysates by in vitro methods (Fritsché and Bonzon, 1990; Jost et al., 1987; Nakamura et al., 1993a,b). Otani et al. (1989b) have shown that the epitope structures of milk proteins may not be identical for man and laboratory animals. Moreover, in some studies (Nakamura et al., 1993a,b) IgG residual antigenicity has been used as a measure of IgE residual antigenicity. However, a correlation between IgE and IgG antibodies has not been found for all milk proteins (Otani et al., 1989a). Residual antigenicity should, therefore, preferentially be determined by use of human antibodies.

CONCLUSIONS

REDUCTION OF WHEY PROTEIN ANTIGENICITY was mainly determined by the molecular structure of the antigenic epitopes and the specificity of the enzyme used for proteolysis. Neither the DH nor molecular mass distribution of hydrolysates reflected the degree of antigenicity of hydrolysates of the individual whey proteins. Those parameters were not suitable for assessing decreases in antigenicity. Enzymatic proteolysis may also increase protein antigenicity by exposing more antigenic sites during hydrolysis as observed for BSA and B-IgG. Antigenic sites of individual whey proteins BSA and B-IgG were completely eliminated by combination of pepsin and Corolase PP but were hardly affected by Corolase 7092. Intact epitopes of αLa and βLg remained present in both hydrolysates. These enzymes did not result in completely hypoallergenic hydrolysates. A final ultrafiltration step was a prerequisite. A filter with molecular mass cut-off 3000 Da decreased residual antigenicity of the Corolase 7092 WPC hydrolysate, but had little effect on the pepsin/Corolase PP hydrolysate. Definite conclusions concerning allergenicity of protein hydrolysates, require *in vivo* experiments. Residual antigenicity of WPC after hydrolysis with enzymes, different from those of the digestive tract could be further reduced or eliminated during simulated human digestion.

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Skimmilk Solids as Substrate for the Preparation of Casein Enzymatic Hydrolysates

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ABSTRACT

The time-course hydrolysis curves of low-heat (LH) and high-heat (HH) skimmilk powders (SMP) by trypsin or chymotrypsin were compared to that of sodium caseinate (SC). Rates of hydrolysis, shown to be different ($P < 0.05$), were SC > LH-SMP > HH-SMP. A two-step ultrafiltration process was applied to hydrolyzed LH-SMP and to hydrolyzed SC, and results were compared. Protein and lactose composition differences between LH-SMP and SC were accentuated by ultrafiltration. The hydrolysis profile (molecular weight distribution of peptides, amino acid content) of LH-SMP tryptic hydrolysate showed a greater proportion of short peptides (<2000 Daltons) and a lower content in GLU, PRO, VAL and TYR, compared to that of SC.

Key Words: casein, skimmilk powder, enzyme hydrolysate, trypsin

INTRODUCTION

INDUSTRIAL PRODUCTION OF CASEIN HYDROLYSATES was initiated in the late 70s, for preparation of products designed for intravenous or oral clinical nutrition of patients with protein digestion disorders, or following surgery of the digestive tract (Manson, 1980). Evidence has shown that some specific peptidic sequences in α_{s1} and β-caseins could also be considered as bioactive peptides such as opioids or mineral carriers in human metabolism (Maubois and Brulé, 1982; Meisel and Schlimme, 1990).

Casein hydrolysates are generally prepared from sodium caseinate (Brulé et al., 1980) that is hydrolyzed by proteolytic enzymes such as pancreatin. The hydrolysate is further ultrafiltered to eliminate the enzyme and high-molecular-weight peptides from the mixture, and thereby a product enriched in short peptides results. In this process, the composition and the physicochemical state of the substrate (the caseins) represent important factors for the effectiveness of enzymatic hydrolysis. Given that the preparation of sodium caseinate involves several steps (acid precipitation, washing of precipitate, neutralization, etc.), it may be beneficial to use skimmilk solids as a substrate for producing casein hydrolysate. However, the use of milk solids could cause undesirable changes in composition and properties of the final product, since their preparation involves heat treatments which favor whey protein-casein interactions.

The milk system is characterized by a heterogeneous composition in which caseins account for 25–28% of total solids (Walstra and Jenness, 1984). Lactose represents the major component of skimmilk on a dry basis (55–60%), followed by proteins (33–36%) and ash components (8–10%). The natural pH range of milk is 6.6–6.8, its calculated ionic strength is 0.08, and the presence of phosphate and citrate salts in milk are responsible for its buffering capacity. Casein represents 76–86% of the total protein content of milk (Swaisgood, 1992); the remaining fraction consists of soluble proteins β-lactoglobulin, α-lactalbumin, bovine serum albumin, immunoglobulins and proteoses-peptones. In its native form, casein exists as colloidal particles (30–300 nm, casein micelles) which are protein aggregates linked together by interactions by calcium phosphate (Rollema, 1992). The internal structure of casein micelles has not been elucidated, but researchers generally agree that micellar casein exists as aggregates or submicelles of α_s- β- and κ-casein supported by hydrophobic interactions and salt bridges (Walstra, 1990). The submicelles or aggregates are presumably linked together by colloidal calcium phosphate (CCP) that is covalently bonded to phosphorylation centers of α_s and β caseins (Holt et al., 1986). Casein micelles are known to show dynamic equilibrium with milk salts and soluble caseins. The presumed degree of organization of the micelle structure introduces questions about the susceptibility of caseins in the micellar state to enzymatic hydrolysis.

Our objective was to investigate the effect of using skimmilk solids as a substrate for casein hydrolysis. In the first experiment, the effect of a pre-heat treatment in manufacture of skimmilk powder (low-heat and high-heat) on tryptic and chymotryptic hydrolysis profiles was evaluated. These results were compared with sodium caseinate as reference, in order to select the most suitable substrate. Sodium caseinate and the selected substrate were then hydrolyzed with trypsin and chymotrypsin using a two-step ultrafiltration process described by Turgeon and Gauthier (1990). Finally, hydrolysate fractions from this process were characterized for overall composition, molecular weight distribution profile of peptidic components, and amino acid contents.

MATERIALS & METHODS

Materials

Low-heat (LH; 76°C/16 sec) and high-heat (HH; 88°C/21 min) skimmilk powders (SMP) containing, respectively, 34.6 and 35.2% (w/w) proteins, were obtained from Agrinove (Ste. Claire, Quebec). Sodium caseinate (85.1% w/w protein) was purchased from ICN (Cleveland, OH). Trypsin (Type III-S) and chymotrypsin (Type II) from bovine pancreas, ATEE (N-acetyl-L-tyrosine ethyl ester), BAEE (N-α-benzoyl-L-arginine ethyl ester), o-phthaldialdehyde (OPA), L-leucine, L-leucyl-L-leucyl-L-Leucine, polymixin-β-sulfate, β-chain of insulin, ribonuclease A, ovalbumin and sodium dodecyl sulfate (SDS) were obtained from Sigma Chemical Company (St. Louis, MO). All other products were reagent grade.

Specific activity of enzymes

Enzyme activities were determined spectrophotometrically according to the method of Schwert and Takenaka (1955) using N-acetyl-L-tyrosyl ethyl ester (ATEE) and N-α-benzoyl-L-arginine ethyl ester (BAEE) as substrates for chymotrypsin and trypsin, respectively. Trypsin and chymotrypsin had 11 280 BAEE and 102 ATEE units of specific activity (per mg protein), respectively.

Time-course hydrolysis of substrates

Tryptic and chymotryptic hydrolysis profiles of different substrates were determined in triplicate at laboratory scale. Substrates were reconstituted in distilled water (200g of 3.5% w/w protein solution, pH 8.0, 40°C), and 0.5-mL samples were taken for determination of the initial content of α-amino nitrogen by the *o*-phthaldialdehyde (OPA) method (Church et al., 1983). The hydrolysis reaction was initiated by adding 5 mL trypsin or chymotrypsin solution (0.7% w/v prepared in 0.001N HCl) to yield a final enzyme:substrate (E:S) ratio of 1:200. The hydrolysis was carried out for 45 min, and the pH was maintained at 8.0 by addition of 0.5N NaOH.

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The degree of hydrolysis (DH), defined as percentage of peptide bonds cleaved, was determined using the pH-stat technique (Adler-Nissen, 1986) and the OPA method (Church et al., 1983) concurrently. For the pH-stat technique, DH was calculated from $DH = h/h_{tot} \times 100\%$ where h_{tot} refers to the total number of peptidic bonds, based on the amino acid composition of the proteins and h refers to the number of peptidic bonds cleaved. This latter parameter is proportional to the base consumption during hydrolysis:

$$h = B \times N_b \times \frac{1}{\alpha} \times \frac{1}{MP}$$

where B is the base consumption (mL), N_b is the normality of the base, MP is the mass of protein (g), and $1/\alpha$ is the average degree of dissociation of α-NH_2 residues released during hydrolysis.

For the OPA method, samples (0.5 mL) were collected during the reaction and immediately diluted in 5 mL of 1% SDS to inactivate the enzymes. The number of free α-NH_2 groups was determined spectrophotometrically using OPA reactant and L-leucine as standards. For both methods, the total content of peptidic bonds (h_{tot}) used for the DH calculations were 8.2 meqv.g^{-1} for sodium caseinate and skimmilk powders, respectively (Adler-Nissen, 1986).

Enzymatic hydrolysis of substrates with the two-step ultrafiltration process

The procedure described by Turgeon and Gauthier (1990) was applied, using the enzymatic conditions previously described but adjusted for the following larger-scale process: 100 kg of 3.5% w/w protein solution, pH 8.0, 40°C, E:S ratio of 1:200, constant pH adjustment during hydrolysis by addition of 4N NaOH. After 45 min hydrolysis, proteolytic products were removed continuously by ultrafiltration (UF) using two membranes with nominal molecular weight cut-off of 30,000 Daltons (HF26-5-43-PM30, Romicon Inc., Woburn, MA). The hydrolysates were concentrated to 3× volume concentration factor and diafiltered using two diavolumes of water. The permeate and retentate of the first UF were referred to as total hydrolysate (TH) and reaction mixture (RM), respectively. TH was further fractionated by a second ultrafiltration (4× concentrated) using smaller cut-off membranes of 1,000 Daltons (two HF26-5-43-PM1, Romicon Inc., Woburn, MA) to remove amino acids and small peptides. The retentate of the second UF was referred to as mixture of polypeptides (MP), and the permeate was referred to as mixture of amino acid and small peptide fractions (AA). These fractions (RM, TH, MP, AA) were frozen, lyophilized and stored at room temperature (≈23°C) until further analysis. Each treatment was carried out in duplicate.

Chemical analysis

Protein content (N × 6.38) of substrate and hydrolysate fractions was determined in duplicate by the Kjeldahl method (IDF, 1986) using a Büchi block digestor 430 equipped with a distillation unit 321 (Brinkman Instrument, Montreal). Lactose values were obtained in triplicate using the method of Marier and Boulet (1959). Moisture content was determined in duplicate by the AOAC (1980) method and fat in triplicate by the Mojonnier method (AOAC, 1980). For ash content, samples were charred and then ignited overnight in a muffle furnace at 550°C. After cooling, H_2O_2 (2 mL) was added to these samples, and they were heated until the ash was white and then ignited overnight at 550°C.

Calcium, potassium and sodium contents were determined by atomic absorption spectroscopy (Ca) and emission spectroscopy (K, Na) (Instrumentation Laboratory spectrophotometer, Model 1L751, Wilmington, MA). Total phosphorus was measured in triplicate by the colorimetric method described by Allen (1940).

Molecular weight (MW) distributions of protein components in tryptic and chymotryptic hydrolysates were determined by high-performance size-exclusion chromatography (HPSEC, LKB System) using a TSK-2000SW column (Vijayalakshmi et al., 1986). Protein standards were L-leucyl-L-leucyl-L-leucine, polymixin-β-sulfate, β-chain of insulin, ribonuclease A and ovalbumin. To compare the MW distribution of hydrolysates, the total area of each chromatogram was integrated, separated into three ranges of molecular weight (>5000, 2000–5000, <2000 Daltons), and expressed as percentage of total area.

The amino acid composition of the hydrolysate fractions was determined with a high-performance amino acid analyzer system (6300 Beckman, Palo Alto, CA), following sample hydrolysis (6N HCL, 100°C, 24 hr).

Statistical treatment

Statistical treatment of enzymatic hydrolysis profiles from the different substrates was performed by a multiple regression model after linearizing data by a logarithmic transformation (Neter et al., 1985).

RESULTS & DISCUSSION

Effect of pre-treatment of substrates on their time-course hydrolysis

The time-course hydrolysis (DH vs time) was obtained (Fig. 1) with trypsin (A, C) and chymotrypsin (B, D) for the three substrates: low-heat skimmilk powder (LH-SMP), high-heat skimmilk powder (HH-SMP), and sodium caseinate (SC). The degree of hydrolysis (DH) was determined by the pH-stat technique (Fig. 1A and B), or by the OPA method (Fig. 1C and D). As reported by Turgeon et al. (1991), DH values obtained with the OPA method were lower (≈35%) than those obtained with the pH-stat technique. This may be associated with an overestimation of the $1/\alpha$ factor used in the DH calculation of the pH-stat technique. With this latter method (A, B), no statistically significant differences ($P < 0.05$) were found between hydrolysis profiles of the three substrates. As for the pH-stat technique, DH values obtained with the OPA method were higher for trypsin (4.8–6.0%) than for chymotrypsin (3.2–4.1%). This could be related to differences in enzyme specificity and in amounts of target amino acids in the proteins. Trypsin is highly specific for peptide bonds containing carboxyl groups (arginine and lysine) while the specificity of chymotrypsin is towards aromatic amino acids (tyrosine, phenylalanine and tryptophan).

Despite the higher variability of data with the OPA method, hydrolysis profiles of the 3 substrates were different ($P < 0.05$) and rates of hydrolysis were SC > LH-SMP > HH-SMP for both enzymes. These results should only be interpreted after taking into account the different treatments involved in preparation of the substrates. For LH-SMP and HH-SMP, the preheat treatment (76°C – 16 sec. vs 83°C – 21 min) was known to induce interactions between β-lactoglobulin (β-lg) and κ-casein via disulfide-sulfhydryl exchanges, and they increase with temperature (Swaisgood, 1992). These interactions could reduce the susceptibility of SMP to proteolysis, compared to sodium caseinate. It is a β-lg free substrate in which caseins exist in a dissociated molecular state, and it is probably more available for enzyme reactions. Nevertheless, the hydrolysis profile of LH-SMP was the most similar to that of sodium caseinate ($P < 0.05$), and LH-SMP was selected for larger-scale hydrolysis.

Though DH values (Fig. 1) obtained for SMP (LH and HH) were lower than for SC, the molecular weight distribution of protein components in the 45-min hydrolysates (Table 1) indicated a higher production of short peptides (<2000 Daltons) from SMP than from SC. This could be related to the presence of whey proteins in SMP and to the different distribution of target amino acids in their primary sequence. As for DH values, the pre-heating treatment of SMP affected the hydrolysis profile, and led to a lower amount of short peptides (<2000 Daltons) in tryptic and chymotryptic hydrolysates of HH-SMP compared to those from LH-SMP.

Effect of substrates on composition of hydrolysate fractions obtained with two-step ultrafiltration

Four distinct hydrolysate fractions were obtained from the 2-step ultrafiltration (UF) process of Turgeon and Gauthier (1990): reaction mixture (RM), total hydrolysate (TH), mixture of polypeptides (MP) and amino acid and small peptides fraction (AA). Low-heat skimmilk powder (LH-SMP) was used as the substrate, and it was compared to sodium caseinate (SC). Preliminary analyses of the two protein substrates (Table 2) indicated that LH-SMP differed from SC by lower protein (35.7% vs 92.6%), higher lactose (57.2% vs 0.8%) and higher ash (8.1% vs 4.4%) content. Moreover, LH-SMP contained 1624 mg/100

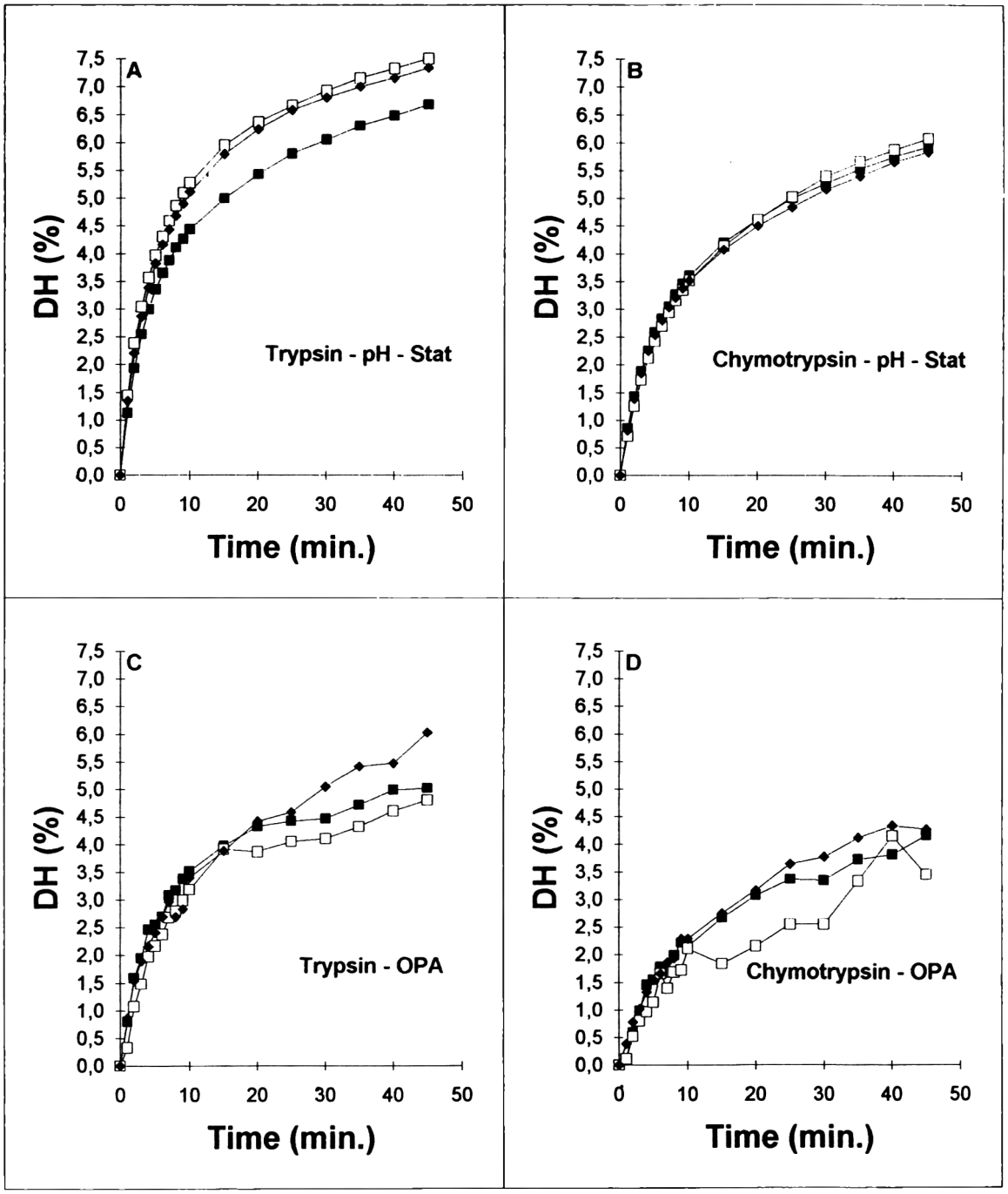


Fig. 1—Time-course hydrolysis of sodium caseinate (♦), low-heat (■) and high-heat (□) skimmilk powders obtained with trypsin (A, C) and chymotrypsin (B, D). Degree of hydrolysis (DH) was determined with pH-stat technique (A, B) or OPA method (C, D).

Table 1—Molecular weight distribution[a] (%) of protein components in tryptic and chymotryptic hydrolysates from low-heat (LH) and high-heat (HH) skimmilk powders (SMP) and sodium caseinate (SC)

Molecular wt (Daltons)	Trypsin			Chymotrypsin		
	LH-SMP	HH-SMP	SC	LH-SMP	HH-SMP	SC
> 5000	5.5	9.7	10.3	7.6	8.5	13.3
5000 - 2000	17.9	20.0	20.0	18.6	19.6	21.5
< 2000	76.6	70.3	69.7	73.8	71.9	65.2

[a] Molecular weight distribution was calculated by integrating the total area of the chromatogram. The total surface was separated into three ranges of molecular weight, and each was expressed as percentage of the total area.

Table 2—Total composition of low-heat skimmilk powder (LH-SMP) and sodium caseinate (SC)[a]

Components (% of dry matter)	Substrate	
	LH-SMP	SC
Protein	35.7	92.6
Lactose	57.2	0.8
Lipid	0.7	1.4
Ash	8.1	4.4
Ca (mg/100g)	1624	225
Na (mg/100g)	506	2595
K (mg/100g)	1736	32
P (mg/100g)	956	786

[a] From Pouliot et al., 1993.

g of Ca, and 3828 mg/100 g of P, compared to SC which contained 225 mg/100 g of Ca, and 1543 mg/100g P, respectively. However, it had a much lower Na content (506 vs 2595 mg/100 g) than SC. Overall composition were compared for the four hydrolysate fractions obtained after ultrafiltration of tryptic (Table 3) or chymotryptic (Table 4) hydrolysates of LH-SMP and SC. The compositional differences observed between both sub-

Table 3—Comparative composition and degree of hydrolysis (DH) of hydrolysate fractions (RM, TH, MP, AA) obtained from tryptic hydrolysis of low-heat skimmilk powder (LH-SMP) or sodium caseinate (SC)[a] using the two-step ultrafiltration process

Components	RM		TH		MP		AA	
(% of dry matter)	LH-SMP	SC	LH-SMP	SC	LH-SMP	SC	LH-SMP	SC
Protein	62.8	84.3	28.5	94.9	49.5	95.0	24.0	95.2
Lactose	12.0	1.6	69.6	0.6	44.9	0.3	71.0	0.2
Lipid	2.9	4.5	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1
Ash	19.5	7.9	7.1	6.2	6.2	6.6	7.0	5.9
Ca (mg/100g)	5250	667	182	146	180	207	196	27
Na (mg/100g)	770	2549	1200	3054	1225	2447	1288	2716
K (mg/100g)	1019	30	1662	41	1715	29	1888	36
P (mg/100g)	3453	1397	448	560	425	800	436	298
DH[b] (%)	0.8	3.3	14.2	10.4	5.9	6.1	19.4	17.8

[a] From Pouliot et al., 1993.
[b] Degree of hydrolysis (DH) was determined by the OPA method.

Table 4—Comparative composition and degree of hydrolysis (DH) of hydrolysate fractions (RM, HT, MP, AA) obtained from chymotryptic hydrolysis of low-heat skimmilk powder (LH-SMP) or sodium caseinate (SC)[a] using the two-step ultrafiltration process

Components	RM		TH		MP		AA	
(% of dry matter)	LH-SMP	SC	LH-SMP	SC	LH-SMP	SC	LH-SMP	SC
Protein	63.7	89.3	28.9	95.9	50.9	99.0	24.1	94.2
Lactose	12.0	1.6	69.0	0.4	43.4	0.3	69.3	0.2
Lipid	3.3	4.9	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1
Ash	6179	8.2	6.6	5.8	5.8	5.5	7.1	6.9
Ca (mg/100g)	716	753	183	85	216	222	264	16
Na (mg/100g)	962	2626	1271	2525	1118	2480	1301	2917
K (mg/100g)	3826	35	1879	32	1617	34	1879	37
P (mg/100g)		1543	406	475	366	652	413	262
DH[b] (%)	1.9	6.5	9.7	7.0	5.6	4.8	12.0	11.3

[a] From Pouliot et al., 1993.
[b] Degree of hydrolysis (DH) was determined by the OPA method.

strates (Table 2) were intensified by UF fractionation of hydrolysates, because of the high protein retention of the membranes (≥80%), compared to lactose (0%) (Pouliot et al., 1993).

A similar relationship was observed for both enzymatic hydrolysates: the fractions from SC always contained more protein (≥84%), compared to LH-SMP fractions which ranged from 24% to 64%. The lactose content of SC fractions was consistently very low (0.2–1.6%), compared to LH-SMP fractions (12–71%). Similar rejection properties regarding Ca and P were observed for both substrates. Results are reported on a dry basis thus relationships observed with LH-SMP for lactose and proteins were accentuated and should be carefully considered. For example, the free passage of lactose through the ultrafiltration membranes combined with the simultaneous rejection of protein would increase the apparent protein content on the retentate side as it decreased this value on the permeate side.

The degree of hydrolysis (DH) measured by the OPA method indicated differences between hydrolysate fractions obtained from trypsin (Table 3) and chymotrypsin (Table 4) and between both substrates. The RM fractions showed higher DH values for chymotrypsin than for trypsin and much greater DH values for sodium caseinate (SC) than for LH-SMP. Differences between substrates were still relatively proportional for tryptic (3.3% for SC vs 0.8% for LH-SMP) and chymotryptic hydrolysates (6.5% for SC vs 1.9% for LH-SMP). However, such differences gradually decreased as fractionation proceeded: for AA fractions, the DH values for tryptic hydrolysates were 19.4% vs 17.8%, and for chymotryptic hydrolysates the DH values were 12.0% vs 11.3% for LH-SMP and SC, respectively. These results suggest that, although the two substrates were hydrolyzed differently, the use of ultrafiltration membranes may provide similar fractions based on content of free amino groups. This effect is related to the selectivity of the membranes and has been reported (Pouliot et al., 1993).

The molecular weight distributions of protein components were compared from MP and AA fractions of tryptic or chymotryptic hydrolysates obtained from LH-SMP and SC (Table 5). The AA fractions obtained with trypsin showed differences related to the nature of the substrate: LH-SMP had 87.9% short peptides (<2000 Daltons) compared to 79.5% for SC. These data were consistent with respective DH values (Table 3) of 19.4% (LH-SMP) and 17.8% (SC) and with molecular weight distributions of protein components before UF fractionation (Table 1). Tryptic hydrolysis of LH-SMP apparently produced more short peptides than from SC, although its absolute level of proteolysis (Fig. 1C) was lower. As mentioned, the presence of whey proteins in SMP and the different distribution of target amino acids (LYS, ARG) in their primary sequence could account for this.

For chymotryptic hydrolysates, the molecular weight distribution of protein components showed a somewhat different result. Differences were also found in MP fractions where LH-SMP showed a higher level of low-range peptides (<2000 Daltons) compared to SC (69.6% VS 64.5%), while the content of short peptides in AA fractions was similar (79%) for both substrates. The higher content of chymotrypsin-target amino acids in SC, compared to LH-SMP, could explain the higher extent of hydrolysis of SC.

Effect of substrates on amino acid profiles of hydrolysate fractions

Because of different protein compositions, LH-SMP and SC were expected to exhibit different amino acid profiles. The amino acid profiles of the two substrates were compared (Fig. 2). Sodium caseinate (SC) contains a greater proportion of glutamic acid (GLU), proline (PRO), valine (VAL), phenylalanine (PHE) and tyrosine (TYR) compared to LH-SMP. This could be explained by the fact that serum proteins, which are present in LH-SMP, contained lesser amounts of these amino acids (Pelissier, 1984). Amino acid profiles of the hydrolysate fractions (TH, MP, AA) obtained following tryptic (Fig. 3) or chymotryptic (Fig. 4) hydrolysis of LH-SMP and SC were also compared. Two effects must be considered in interpretation of these results: (1) the effect of membrane retentivity, and (2) the effect of enzyme specificity.

In a previous study (Pouliot et al., 1993), polysulfone membranes were shown to specifically reject polar amino acids, but they did not affect the content of nonpolar amino acids. Also,

Table 5—Molecular weight distribution[a] (%) of protein components in the hydrolysate fractions (MP, AA) obtained from tryptic or chymotryptic hydrolysis of low-heat skimmilk powder (LH-SMP) and sodium caseinate (SC)

Molecular wt (Daltons)	Trypsin				Chymotrypsin			
	MP		AA		MP		AA	
	LH-SMP	SC	LH-SMP	SC	LH-SMP	SC	LH-SMP	SC
> 5000	3.7	5.9	0.1	3.1	2.9	4.1	1.8	1.4
5000 - 2000	28.0	28.9	12.0	17.4	27.5	31.4	19.5	19.7
< 2000	68.3	65.2	87.9	79.5	69.6	64.5	78.7	78.9

[a] Molecular weight distribution was calculated by integrating the total surface of the chromatogram. The total surface was separated into three ranges of molecular weight and each was expressed as percentage of the total surface.

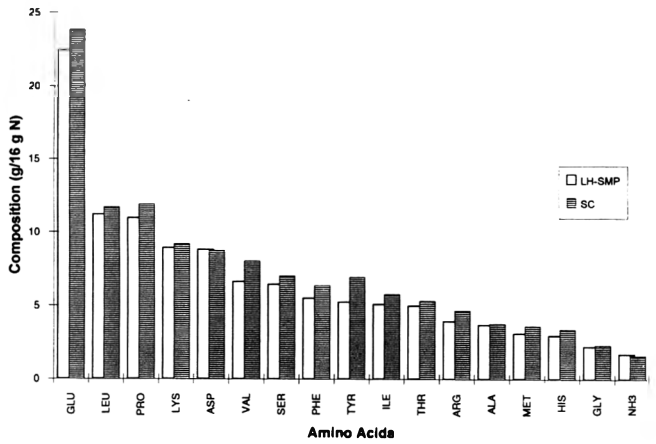


Fig. 2—Comparative amino acid profile of low-heat skimmilk powder (LH-SMP) and sodium caseinate (SC). Results for SC were adapted from Pouliot et al., 1993.

most of the target amino acids of enzymes are generally found in the permeates (AA), since they appear in greater amounts in the AA fractions (Fig. 3) of tryptic hydrolysates. Also, PHE and TYR, the target amino acids of chymotrypsin, are found in greater amounts in the AA fractions (Fig. 4) of chymotryptic hydrolysates. For both enzymes (Fig. 3 and 4), the major difference between MP and AA fractions was the amount of polar amino acids such as GLU and ASP. These two amino acids represent the effects of polysulfone on polar amino acids, since they are not involved in the hydrolysis mechanism of trypsin or chymotrypsin.

The effects related to specificity of the two enzymes on LH-SMP and SC cannot be directly identified from these profiles. More study should be given to the TH fractions obtained from each substrate with the two enzymes, since this fraction contains the majority of peptides released following the 45-min proteolysis. More information about the nature of the protein could be obtained by comparing profiles of TH fractions from each substrate.

The overall effects of using LH-SMP as a substrate for casein hydrolysis on the amino acid profile of the fractions were summarized (Table 6). The relationships of the profiles was calculated using the Marchalonis and Weltman (1971) parameter SΔQ which is defined as $\Sigma (X_{ij} - X_{kj})^2$, where X_{ij} refers to the content of amino acid (J) in reference sample (I), and X_{kj} refers to the content of amino acid (J) in the sample (K) under study. The calculation demonstrated that LH-SMP appeared to be similar to CS, as shown by its low SΔQ value (2.92). As well, the SΔQ parameter increased after UF-fractionation TH < MP < AA, indicating that membrane separation modified the amino acid profiles of the substrates. However, all values were generally relatively low (≤46.60). These increases in SΔQ could be due to specific rejection of modified or hydroxylated amino acids and to preferential passage of target amino acids for trypsin or chymotrypsin.

CONCLUSION

USE OF TOTAL MILK SOLIDS, in the form of skimmilk powder, can be considered a suitable substrate for preparation of casein

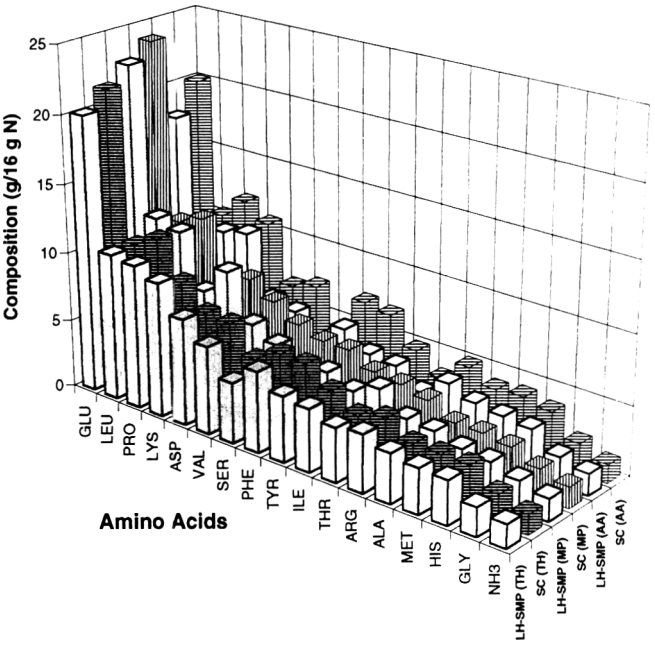


Fig. 3—Amino acid profiles of the hydrolysate fractions (TH, MP, AA) obtained from tryptic hydrolysis of low-heat skimmilk powder (LH-SMP) and sodium caseinate (SC). Results for SC were adapted from Pouliot et al., 1993.

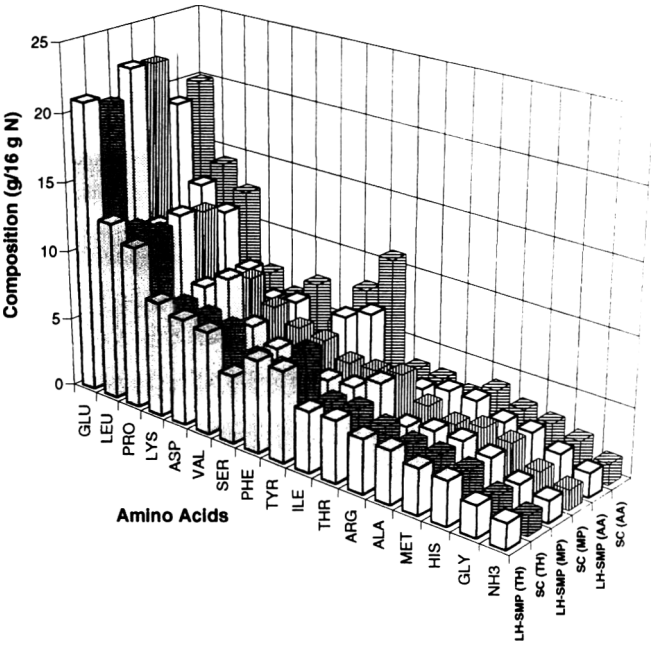


Fig. 4—Amino acid profiles of the hydrolysate fractions (TH, MP, AA) obtained from chymotryptic hydrolysis of low-heat skimmilk powder (LH-SMP) and sodium caseinate (SC). Results for SC were adapted from Pouliot et al., 1993.

Table 6—Marchalonis and Weltman parameter SΔQ[a] for the substrates and UF fractions obtained from tryptic and chymotryptic hydrolysis of CS and LH-SMP

		UF Fractions					
		Tryptic			Chymotryptic		
Substrates		TH	MP	AA	TH	MP	AA
CS	—	3.11	5.10	17.82	10.23	4.62	46.60
LH-SMP	2.92	8.13	12.20	22.02	8.02	12.54	24.08

[a] $S\Delta Q = \Sigma (X_{i,j} - X_{k,j})^2$, where $X_{i,j}$ refers to the content of amino acid (J) in reference substrate (I) and $X_{k,j}$ refers to the content of amino acid (J) in the UF fraction under study (K).

enzymatic hydrolysates. However, additional upstream or downstream purification steps (diafiltration) are necessary to lower lactose and ash. The presence of whey proteins in the substrate is likely to modify the molecular weight profiles of peptides following proteolysis by trypsin; but for chymotryptic hydrolysis, such modifications would be very minor. The use of skim-milk solids appeared to induce very minor modifications in amino acid profiles. However, the effects should not influence the amino acid profiles of UF fractions to a great extent.

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Stability of β-Galactosidase from *Aspergillus oryzae* and *Kluyveromyces lactis* in Dry Milk Powders

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ABSTRACT

A milk-based beverage powder with 2% vegetable fat and reduced levels of lactose is needed by some segments of the consumer market as well as military units. To provide such, two commercial β-galactosidases were dry-blended into milk powders made with coconut, cottonseed, canola or sunflower oils and stored at −20, 4, 25, and 45°C. Enzyme activity was measured monthly by reconstituting the powders and measuring freezing point depression after incubation at 32°C (optimum for β-galactosidase from *Kluyveromyces lactis*) and incubation at 50°C with pH adjusted to 5.0 (for activity of *Aspergillus oryzae* enzyme). Both enzymes retained >95% of original activity at −20 and 4°C. Activity of *K. lactis* enzyme declined moderately at room temperature (≈23°C) and rapidly at 45°C. The *A. oryzae* enzyme retained 93.4% of original activity after 6 mo at 45°C.

Key Words: galactosidase, *Kluyveromyces lactis*, *Aspergillus oryzae*, dry milk, vegetable fat

INTRODUCTION

EFFORTS TO INCLUDE NONFAT DRY MILK as a beverage powder in dried field rations have been mostly unsuccessful. Much of it is not consumed, either because the flavor is unacceptable or because of lactose intolerance. A reduced-lactose milk-based beverage powder would provide the nutritional advantages of skim milk and unsaturated vegetable fat when reconstituted. Hydrolysis of lactose to glucose and galactose prior to drying leads to excessive browning and burning from sticking to hot surfaces in the dryer. Another possible approach would be to incorporate β-galactosidase enzymes into the dry fat-containing powder. The stability of the enzymes in dry form under highly variable storage conditions is not known.

β-Galactosidases are produced by a variety of plant, animal, and microbial cells. Commercially available enzymes are derived from yeasts such as *Kluyveromyces lactis* and fungi such as *Aspergillus oryzae* and *A. niger*. The *K. lactis* enzyme has an optimum pH near that of milk and exhibits appreciable activity at 4–6°C (Nijpels et al., 1980) making it suitable for addition to milk before consumption. The *A. oryzae* enzyme has a pH optimum of 5.0 and an optimum temperature of 50–55°C (Park et al., 1979), making it effective in the acid environment of the stomach.

Rosado et al. (1984) noted that *in vitro* hydrolysis of lactose by addition of β-galactosidase before consumption is not practical when refrigeration facilities are not available. They found *in vivo* hydrolysis with *A. niger* β-galactosidase to be partially effective, however. Barillas and Solomons (1987) demonstrated that β-galactosidase derived from *A. oryzae*, orally administered, was effective in preventing lactose malabsorption in children. Situations in which such milk powders would be used might include field use, where no refrigeration is available, and large-scale feeding operations where overnight refrigeration would be common. In the first situation, the *A. oryzae* enzyme would be effective; in a situation where pre-hydrolysis during overnight refrigeration was possible, the *K. lactis* enzyme would be more effective. Therefore, both enzymes were incorporated for study.

Our objectives were to establish processing conditions to prepare such beverage powders and determine stability of the *K. lactis* and *A. oryzae* enzymes during storage at temperatures which might be encountered in field use. We also evaluated possible effects of different vegetable oils on enzyme stability.

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MATERIALS & METHODS

Enzymes

β-Galactosidase enzymes from *K. lactis* (22,200 Neutral Lactase Units/g) and *A. oryzae* (112,000 Food Chemicals Codex Lactase Units/g) were obtained in powder form from Lactaid Inc., Pleasantville, NJ. The level of addition of the *K. lactis* enzyme (0.858 mg/g powder) was determined by the amount necessary to give 80% hydrolysis in 24 hr at refrigeration temperatures. The amount of *A. oryzae* enzyme used (1.52 mg/g powder) was equivalent to one Lactaid™ caplet per 240 mL serving of milk-based beverage.

Preparation of milk powders

Raw skim milk was pasteurized at 76.7°C for 15 sec and evaporated at 43.3°C to 42–44% solids. Each batch of concentrate was divided into three portions, warmed to 65.6°C, blended with vegetable oil to yield 2% fat when rehydrated to 11% total solids, and homogenized double-stage at 13,800-3450 kPa. The homogenized concentrate was immediately spray-dried at 88.9–90.6°C (outlet temperature) to moisture content < 3%. Enzymes were dry-blended into powders (1.76g *K. lactis* enzyme and 3.12g *A. oryzae* enzyme/2050 g milk powder) and immediately sealed into cans (size 211 × 300, Can Corp. of America, Blandon, PA) under nitrogen. Lactose content was determined enzymatically using the Boehringer-Mannheim lactose/D-galactose test kit (AOAC, 1990).

Three batches of powders were prepared. In the first two batches, the vegetable oils were coconut (supplied by California Oils Corp., Richmond, CA), canola (supplied by Archer Daniels Midland Co., Decatur, IL), and sunflower (Trisun 95, supplied by SVO Enterprises, Eastlake, OH). In the third batch, cottonseed oil (supplied by Lou Ana Foods, Inc., Opelousas, LA) was substituted for canola oil because of flavor concerns.

Analysis of powders

Each powder was analyzed for moisture content by drying under vacuum for 24 hr at 60°C, and for fat content by the Roese-Gottlieb procedure (Marth, 1978). Initial powders were also analyzed for free fat by the method of Tamsma et al. (1959). Lactose content of each powder was determined by the enzymatic-UV method using the Boehringer-Mannheim Lactose/Galactose kit after deproteinization with Carrez solutions (AOAC, 1990).

Storage

Sealed cans from each batch were stored at −20°C, 4°C, room temperature (25°C), and 45°C for 6 mo. Samples were removed and analyzed for enzyme activity initially and at monthly intervals.

Enzyme activity

Preliminary experiments were conducted to determine hydrolysis conditions which would permit estimation of the activity of each enzyme. For these experiments, a 9% solution of nonfat dry milk was prepared and divided into three 240-mL aliquots. To one of these was added 14.1

Table 1—Hydrolysis of lactose by enzymes incorporated into nonfat dry milk reconstituted to 9% total solids after 2 hr incubation at 50°C, pH 5.0, or 32°C, pH 6.5, or after 24 hr at 4°C[a]

	Percent lactose hydrolysis		
Enzyme	50°C, pH 5 2 hr	32°C, pH 6.5 2 hr	4°C, pH 6.5 24 hr
K. lactis (0.059 mg/mL)	0.3	55.6	65.2
A. oryzae (0.112 mg/mL)	87.6	29.6	25.9
Both enzymes same conc as above	89.7	61.5	64.5

[a] Average of three trials.

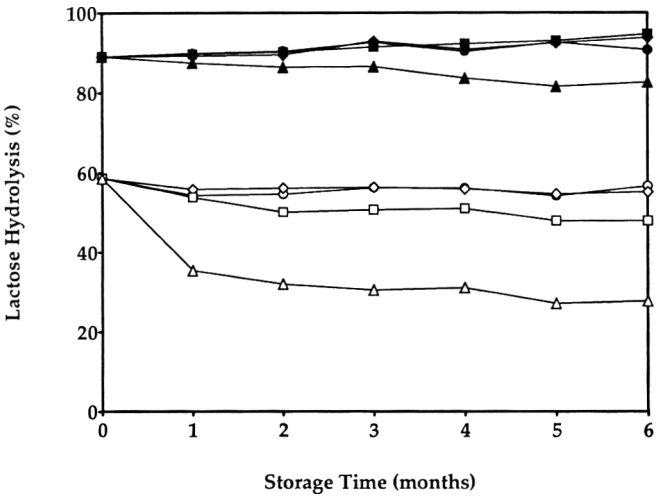


Fig. 1—Effect of storage of milk powders containing *K. lactis* and *A. oryzae* β-galactosidases on lactose hydrolysis in rehydrated products (average of all oils). ▲ 45°C storage, hydrolysis at 50°C, pH 5.0; △ 45°C storage, hydrolysis at 32°C; ■ 25°C storage, hydrolysis at 50°C, pH 5.0; □ 25°C storage, hydrolysis at 32°C; ▼ 4°C storage, hydrolysis at 50°C, pH 5.0; ▽ 4°C storage, hydrolysis at 32°C; ● −20°C storage, hydrolysis at 50°C, pH 5.0; ○ −20°C storage, hydrolysis at 32°C.

mg *K. lactis* enzyme; to a second aliquot, 26.8 mg *A. oryzae* enzyme was added; and to a third aliquot both enzymes were added in the amounts stated. Each of these three samples was divided into three parts: one portion was refrigerated for 24 hr, one portion was incubated at 32°C, while the other was acidified to pH 5.0 and incubated at 50°C. Freezing points were determined initially and after 1 and 2 hr, or after 24 hr for the refrigerated sample, using a Cryette A Automatic Cryoscope (Precision Systems, Inc., Natick, MA). Freezing point depression (FPD) was calculated and converted to percent lactose hydrolysis (described below).

For the storage study, enzyme activity was determined by a similar procedure. For each powder at each time, 22g powder were blended with 178g cold deionized water. The sample was then divided. For one part the pH was adjusted to 5.0 with 2 N HCl; the other part was unadjusted. Aliquots of each portion were used for zero time measurement. The remainder of each portion was divided into test tubes and placed in water baths for incubation. The pH 5 samples were incubated at 50°C for measurement of *A. oryzae* enzyme activity. Unadjusted samples (pH 6.5) were incubated at 32°C for estimation of *K. lactis* activity. Samples were removed after 2 hr and placed in an ice bath. One portion of the pH 6.5 sample was held for 24 hr under refrigeration. Freezing points were determined in triplicate on each sample. Freezing point depression was calculated as the difference between the average freezing point of the incubated sample and the corresponding zero-time sample.

Calculation of percent hydrolysis

FPD data were converted to lactose hydrolyzed (g/100 mL) by dividing by a factor representing the FPD for each 1% lactose hydrolyzed. This factor was determined for each hydrolysis condition (32°C at pH 6.5 and 50°C at pH 5.0) as follows. Representative samples of powder were hydrolyzed as indicated; samples were removed every 30 min, and freezing points determined. A weighed aliquot was diluted and deproteinized immediately to stop the reaction and prepare the sample for measurment of galactose by the enzymatic-UV method described. Hydrolyzed lactose (g/100 mL or percent) was calculated from galactose concentration. The ratio of FPD to hydrolyzed lactose was calculated for each time; these values were averaged for each hydrolysis condition. Percent hydrolysis was calculated from amount of lactose hydrolyzed and total lactose as measured enzymatically in each powder batch.

Statistical analysis

The SAS Software System (SAS Institute, Inc., 1987) was used to evaluate effects of storage temperature and time on enzyme activity in each powder using a general linear model. A Bonferroni T test was used to determine significance of differences between means.

RESULTS & DISCUSSION

ACTIVITY OF β-GALACTOSIDASES has been measured spectrophotometrically using o-nitrophenyl-β-D-galactopyranoside (ONPG) as substrate (Dickson et al., 1979). Also, using lactose as substrate one could measure glucose or galactose produced (Richmond et al., 1981; Kleyn and Trout, 1984). It can also be measured by the freezing point depression cryoscopically in milk or whey (Nijpels et al., 1980; Baer et al., 1980; Chen et al., 1981). Measurement of freezing point depression was chosen as the most suitable for our study because of its speed and accuracy.

The freezing point method was evaluated for use in both neutral and acid products by Baer et al. (1980) and Chen et al. (1981). The accuracy of the method depends on the accuracy of freezing point determinations and the accuracy of lactose/galactose determinations (Chen et al., 1981). We found freezing point measurements were highly reproducible. The enzymatic/UV determinations of lactose and galactose were more variable. However, any error in this procedure should be reflected as a consistent error in the conversion of FPD to percent hydrolysis and would not change relative conclusions regarding enzyme stability.

Results of lactose hydrolysis by the enzymes under conditions for assay were compared (Table 1). Hydrolysis for 24 hr at 4°C was comparable to hydrolysis for 2 hr at 32°C; the latter conditions were reported as a measure of activity of *K. lactis* enzyme. Activity of *A. oryzae* enzyme was not completely eliminated at 32°C or 4°C, but it was sufficiently reduced to permit evaluation of *K. lactis* enzyme activity. When hydrolysis was carried out at 50°C, which at pH 5.0 were optimum conditions for the *A. oryzae* enzyme, activity of the *K. lactis* enzyme was eliminated. These conditions were assumed to provide a measure of the activity of *A. oryzae* enzyme; (they do not, of course, represent true physiological conditions).

Average composition of the milk powders was 19.90% total fat, 39.9% lactose, and 2.76% moisture. Preliminary studies to establish processing conditions involved homogenizing vegetable oils into skim milk before and after condensing. Free fat as a percent of total fat was determined on these powders as a measure of the degree of emulsification achieved. Values of 0.88 to 1.32% indicated excellent emulsification of the added vegetable fats. No difference attributable to sequence of condensation and homogenization was observed. When reconstituted to 11% total solids, the powders produced a beverage containing 2% fat and 9% nonfat milk solids.

The effect of storage temperature on enzyme activity could be seen by comparing percent lactose hydrolysis in all reconstituted powders (Fig. 1). At 32°C, unadjusted pH, levels of hydrolysis >30% must be due to *K. lactis* enzyme activity. Levels of activity < 30% may be due to either enzyme. While incubation at 32°C at the natural pH of milk did not completely eliminate activity of *A. oryzae* enzyme, simple calculation indicates that no *K. lactis* enzyme activity would remain after 6 mo at 45°C. *A. oryzae* enzyme alone could hydrolyze 29.6% of the lactose in 2 hr at 32°C (Table 1); after 6 mo storage at 45°C, this enzyme retained 93.4% of its original activity as measured at 50°C pH 5.0 (Table 2). Multiplying 29.6% by 0.934 gives 27.6% hydrolysis which could be expected as a result of *A.*

Table 2—Retention of original (0 month storage) enzymatic activity after 6 mo storage[a]

Storage (°C)	Percent of original activity	
	32°C, pH 6.5[b]	50°C, pH 5.0[c]
−20	98.4	102.6
4	95.3	105.9
25	83.5	106.0
45	49.4	93.4

[a] Average of all powders.
[b] Values over 50% reflect activity of *K. lactis* enzyme.
[c] Conditions for *A. oryzae* enzyme.

Table 3—Lactose hydrolysis (%) in reconstituted milk powders containing different oils after 6 mo storage; hydrolysis at 32°C, pH 6.5, for *K. lactis* activity

Storage °C	Lactose hydrolysis (%) after 2 hr at 32°C[a]			
	Coconut	Canola	Sunflower	Cottonseed
−20	57.8	58.4	55.1	55.4
4	55.1	56.3	54.7	53.6
25	48.2	48.8	46.9	48.4
45	26.9	27.7	27.2	31.6
no storage	57.8	61.1	59.2	52.9

[a] Averages of all replicates.

Table 4—Lactose hydrolysis (%) in reconstituted milk powders containing different oils after 6 mo storage; hydrolysis at 50°C, pH 5.0, for *A. oryzae* activity

Storage (°C)	Lactose hydrolysis (%) after 2 hr, 50°C, pH 5.0[a]			
	Coconut	Canola	Sunflower	Cottonseed
−20	89.5	96.4	89.8	88.6
4	95.4	94.5	91.2	94.7
25	95.4	97.2	93.8	89.8
45	82.4	83.0	81.7	84.5
no storage	88.8	89.5	89.7	86.9

[a] Averages of all replicates.

oryzae enzyme activity. This is approximately equal to the average percent hydrolysis observed in the powders stored 6 mo at 45°C and hydrolyzed at 32°C (Fig. 1).

Comparison of 32°C with 50°C, pH 5.0, indicated that the *A. oryzae* enzyme was considerably more stable at higher storage temperatures than the *K. lactis* enzyme (Fig. 1). Analysis of variance showed that storage time, temperature, and the interaction of time and temperature significantly affected both assay conditions (32°C/pH 6.5 and 50°C/pH 5.0). Under refrigerated or frozen storage, both enzymes retained ≥ 95% percent of original activity after 6 mo (Table 2). No significant difference occurred between these storage temperatures for either enzyme under any assay conditions used. Most of the deactivation of the *K. lactis* enzyme at 45°C occurred in the first month; loss of activity at room temperature became significant at 3 mo. Activity of the *A. oryzae* enzyme decreased significantly at 5 mo when stored at 45°C. Room temperature storage caused no significant loss after 6 mo. An apparent slight upward trend in activity of *A. oryzae* enzyme was seen (Table 2) as more than 100% retention of activity. This probably was a result of experimental error in pH adjustment or temperature control during the assay, although every effort was made to carefully control such variables.

Tables 3 and 4 show the effect of oil type on enzyme stability when stored for six months at different temperatures. Comparison of the effect of oil type on enzyme stability during storage at 45°C, where differences were greatest, showed relatively minor differences. Differences among coconut, canola, and sunflower oil-containing powders were not significant, but the interaction of oil with storage temperature was significant for the 50°C/pH 5.0 assay. Cottonseed oil was not included in the analysis of variance because only one batch was prepared.

CONCLUSIONS

THE *K. LACTIS* ENZYME would retain appreciable activity if milk powders were stored under nitrogen at 20°C or less, but would lose activity rapidly when stored at 45°C. The *A. oryzae* enzyme would tolerate considerably more temperature abuse when stored under nitrogen. No loss of activity of that enzyme was observed after 6 mo at 25°C, and powders stored at 45°C retained an average 89% of the *A. oryzae* enzyme activity of the same powder stored at 4°C. Therefore incorporation of the *A. oryzae* enzyme can be recommended, but addition of *K. lactis* enzyme would be justified only if the powders could be kept cool.

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Viscosity of Molten Milk Chocolate with Lactose from Spray-Dried Whole-Milk Powders

CARLOS A. AGUILAR and GREGORY R. ZIEGLER

ABSTRACT

Dry whole-milk powders containing 0, 30, 50 and 70% nominal lactose prepared by spray-drying alone, or followed by post-drying crystallization, were incorporated into milk chocolate to give 0–50% substitution of lactose for sucrose. Increasing the concentration of amorphous lactose from spray-dried powders in the chocolates decreased viscosity, increased particle size of refined chocolate mass, and lowered the concentration of surface-active agents at which a minimum in Casson yield value was observed. Increasing the concentration of crystalline lactose from milk powders in the chocolates increased viscosity, decreased particle size, and increased the concentration of surface-active agents at which a minimum in Casson yield value was observed. Conditions which affect lactose crystallinity in milk powders, such as improper storage and handling prior to use in chocolate production, could be responsible for variations in chocolate viscosity noted sometimes by processors.

Key Words: lactose, spray-drying, chocolate, viscosity, surfactants

INTRODUCTION

In July 1986 the Chocolate Manufacturers Association of the USA (CMA) petitioned the Food and Drug Administration (FDA) to amend the standards of identity for milk chocolate and other chocolate products to allow use of lactose and other nutritive sweeteners in the manufacture of milk chocolate (Seguine, 1989; FDA, 1989). Lactose can be added to milk chocolate as a nutritive carbohydrate sweetener (FDA, 1992). Substitution of lactose for sucrose in milk chocolate may provide advantages related to flavor, texture, viscosity and cost.

Milk chocolate is a concentrated particulate suspension with a continuous phase comprising cocoa butter and milk fat, and a dispersed phase comprising cocoa particles, sugar crystals and non-fat milk solids. Crumb-based milk chocolate is prepared by drying a mixture of concentrated milk, sucrose, and cocoa liquor to ca. 98% solids, then adding cocoa butter and lecithin. A dry milk powder-based milk chocolate contains milk powder which is independently dried and then mixed with the remaining ingredients (Heathcock, 1985).

Lactose could be added to milk chocolate in several ways, for example, by direct substitution of crystalline lactose for sucrose, during crumb manufacture, or in the form of high-lactose milk powders. Our investigation considered the substitution of lactose for sucrose in the form of spray-dried, high-lactose milk powders in the manufacture of dry milk powder-based milk chocolate.

The viscosity of molten chocolate following conching (a controlled-temperature mixing process) is standardized by the addition of cocoa butter and surfactants (e.g., lecithin). It is efficient to obtain an appropriate viscosity with a minimum of these two relatively costly components.

Dry whole milk (DWM) accounts for 23% of the total mass in typical milk chocolate, resulting in total lactose between 6 and 8% (Campbell and Pavlasek, 1987; Verhey, 1986). In spray-dried DWM, amorphous lactose forms a continuous matrix in which proteins, fat globules and air cells are dispersed (King, 1965; Buchheim, 1982; Saito, 1985). Amorphous lactose is very hygroscopic, and when exposed to high relative humidity or increased temperature, undergoes an irreversible transition to a crystalline form (Lai and Schmidt, 1990). Lactose crystals in milk powder originate either in the milk concentrate prior to drying or develop after drying (Roetman, 1979). Pre-drying crystallization produces α-lactose crystals of typical "tomahawk" shape. Lactose β-anhydride or α-monohydrate may be formed post-drying depending on storage conditions (Roetman, 1979; Saito, 1985).

Ideally, all of the milk fat should become part of the continuous fat phase during conching. However, only a portion of the total fat in DWM, termed free fat, can be extracted under standardized conditions (Buma, 1971). Zürcher (1976) discussed the suitability of roller- and spray-dried milk powders for chocolate making, and showed a lower consumption of energy and cocoa butter when roller-dried milk powder was used. This difference was attributed to the higher free fat content. Saito (1985) observed the expulsion of fat from DWM accompanying the crystallization of α-lactose monohydrate. Fat expulsion in milk powders during the transformation from amorphous to crystalline lactose may reduce the viscosity of molten milk chocolate.

A major consequence of substituting crystalline lactose for sucrose is the effect on rheological properties of milk chocolate. Hogenbirk (1985) reported that the complete substitution of crystalline lactose for sucrose increased the Casson plastic viscosity and yield value for chocolate processed in a 5-roll refiner. Viscosity and yield value were observed to increase at 50 and 100% substitution when chocolate was processed in a ball mill (Hogenbirk, 1985).

Our objective was to determine the effects of concentration and physical form of lactose (amorphous vs crystalline) in spray-dried milk powders with modified lactose content on the particle size distribution, rheological properties, and utilization of surfactants during manufacture of milk chocolate.

MATERIALS & METHODS

Statistical design and analysis

Data were analyzed in a two-way ANOVA using the milk powder preparation method and the concentration of lactose in the milk chocolates as factors. Two milk powder preparation methods (spray-drying and post-drying crystallization), four lactose concentrations (0, 5, 12 and 28%) in chocolates containing powders prepared by spray-drying, and three lactose concentrations (5, 12 and 28%) in chocolates containing powders prepared by post-drying crystallization were considered. Means were separated by single degrees of freedom tests at a significance level of 0.05 (Neter et al., 1990) using the General Linear Models procedure of SAS® (SAS Institute, Inc., 1985).

Preparation of milk powders

Conventional spray-drying. Spray-dried whole milk powders with 0, 30, 50 and 70% nominal lactose were prepared according to the method of Aguilar and Ziegler (1993a). Lactose-free whole milk concentrate, prepared by ultrafiltration and diafiltration of homogenized, pasteurized whole-milk, was mixed with lactose solution (28–39% w/w depending on the desired lactose content of the final powder) before spray-drying. Milk powders were prepared in duplicate and stored at 20–25°C in sealed plastic containers until used. These materials are referred to as dry whole milk (DWM).

Post-drying crystallization. After spray-drying, milk powders with nominal lactose concentrations of 30, 50 and 70% were exposed to relative humidity of 77% for 7 days (20–22°C) in sealed desiccators to crystallize the lactose (Aguilar and Ziegler, 1993b). Milk powders were

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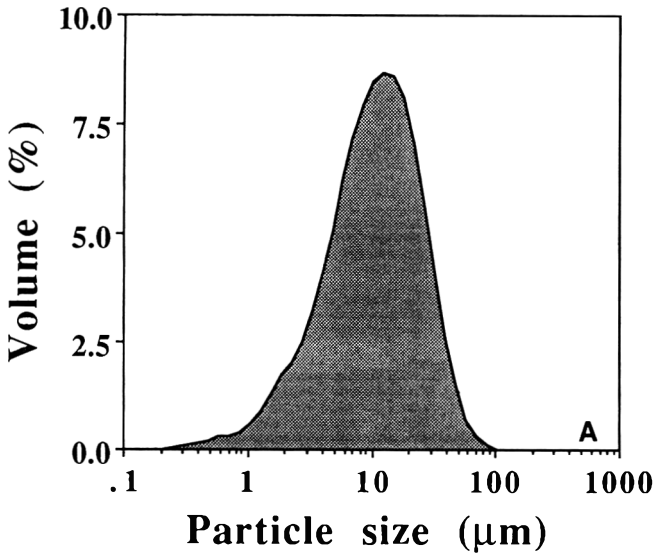


Fig. 1A—Volume-based particle size distribution of refined milk chocolate containing DWM (0% nominal lactose).

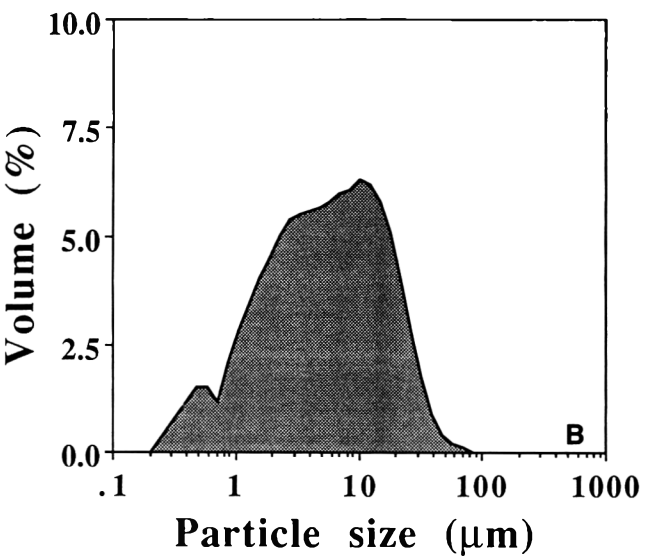


Fig. 1B—Volume-based particle size distribution of refined milk chocolate containing crystallized DWM (28% nominal lactose).

Table 1—Formulations for milk chocolates containing milk powders with differing lactose contents

	Lactose concentration in milk powders (%)				
	36.9[a]	0	27.4	47.7	67.2
Ingredients (g)					
Milk powder[1]	106.2	67.0	92.3	128.1	204.3
Sucrose (granulated)[2]	220.8	260.0	234.7	198.9	122.7
Cocoa butter	99.0	99.0	99.0	99.0	99.0
Cocoa liquor	72.5	72.5	72.5	72.5	72.5
Lecithin	1.5	1.5	1.5	1.5	1.5
Total	500.0	500.0	500.0	500.0	500.0
[1]Milk powder (g)					
Nonlactose	67.0	67.0	67.0	67.0	67.0
Lactose	39.2	0.0	25.3	61.1	137.3
Total	106.2	67.0	92.3	128.1	204.3
Sugars (g)					
[1]Lactose	39.2	0.0	25.3	61.1	137.3
[2]Sucrose	220.8	260.0	234.7	198.9	122.7
Total	260.0	260.0	260.0	260.0	260.0
% Lactose in total sugars	15.1	0.0	9.7	23.5	52.8
% Lactose in chocolate	7.8	0.0	5.0	12.2	27.5

[a] Typical composition of milk chocolate containing commercial dry whole-milk powder. This formula was not evaluated in this study; it is included for comparison purposes only.

prepared in duplicate and stored at 20–25°C in sealed plastic containers until used. These materials are referred to as crystallized dry whole milk (CDWM).

Preparation of milk chocolates

Lactose was partially substituted for sucrose in milk chocolate formulations. Total sugar (lactose + sucrose), cocoa butter, cocoa liquor, lecithin, and non-lactose milk solids were kept constant. Lactose content in the milk powders was determined by AOAC method 984.15 (AOAC, 1990), and used to calculate the amount of DWM or CDWM required to achieve desired levels of lactose and milk solids in each sample (Table 1).

Blended West African cocoa butter and low roast West African liquor were melted (55°C) in a convection oven (Model 1350 F, VWR Scientific, Cleveland, OH). The chocolate ingredients were mixed at room temperature for 30 min (Model N-50, The Hobart Mfg., Inc., Troy, OH) and stored at room temperature in sealed plastic containers until refined. Only 2/3 of the total cocoa butter and no lecithin were added during mixing.

Chocolate mixes (55°C) were refined in two passes using a horizontal 3-roll refiner (F.B. Lehmann Maschinenfabrik, Germany) cooled with tap water (14–17°C). The gap in the refiner was set by processing a

Table 2—Particle size, median, volume-based diameter (d[V, 0.5]), of refined milk chocolates

Nominal lactose in chocolate (%)	Nominal lactose in milk powder (%)	Conventional spray-drying (μm)	Post-drying crystallization (μm)
0	0	9.3 ^{ab}	
5	30	7.3 ^{bA}	9.5 ^{aA}
12	50	9.1 ^{abA}	6.1 ^{bB}
28	70	11.1 ^{aA}	6.3 ^{bB}

[a,b] Within a column, means followed by same superscript not significantly different ($P > 0.05$).

[A,B] Within a row, means followed by same superscript not significantly different ($P > 0.05$).

Table 3—Casson model parameters and apparent viscosity @ 10 s^{-1}

Nominal lactose in chocolate (%)	Nominal lactose in milk powder (%)	Conventional spray-drying	Post-drying crystallization
		Casson yield value (Pa)	
0	0	23.1 ^{a}	
5	30	23.8 ^{aA}	26.3 ^{aA}
12	50	19.3 ^{aA}	58.2 ^{bB}
28	70	11.4 ^{bA}	103.8 ^{cB}
		Casson plastic viscosity (Pa • s)	
0	0	4.56 ^{a}	
5	30	4.32 ^{aA}	3.70 ^{aA}
12	50	4.32 ^{aA}	5.09 ^{bB}
28	70	4.06 ^{aA}	5.36 ^{bB}
		Apparent viscosity @ 10 s^{-1} (Pa • s)	
0	0	14.1 ^{a}	
5	30	13.8 ^{abA}	13.4 ^{aA}
12	50	12.6 ^{bA}	23.3 ^{bB}
28	70	10.0 ^{cA}	33.1 ^{cB}

[a,b,c] Within a column, means followed by same superscript not significantly different ($P > 0.05$).

[A,B] Within a row, means followed by same superscript not significantly different ($P > 0.05$).

chocolate mix containing commercial DWM (Table 1). The median, volume-based particle diameter, d[v,0.5], of this refined mix was 8.0 μm, as determined by laser light-scattering particle size analysis. To avoid exposure to humidity, the refined masses were stored in sealed 2.3 kg plastic containers that, in turn, were placed in sealed 4.6 kg plastic containers with indicating anhydrous calcium sulfate (Fisher Scientific, Pittsburgh, PA).

Chocolates were conched for 4 hr (55–57°C) in a Hobart mixer (Model N-50, The Hobart Mfg., Co., Troy, OH) equipped with a jacketed bowl and constant temperature, recirculating water bath (Haake model D8; Fisons Instruments. Valencia, CA). The refined mass was weighed and

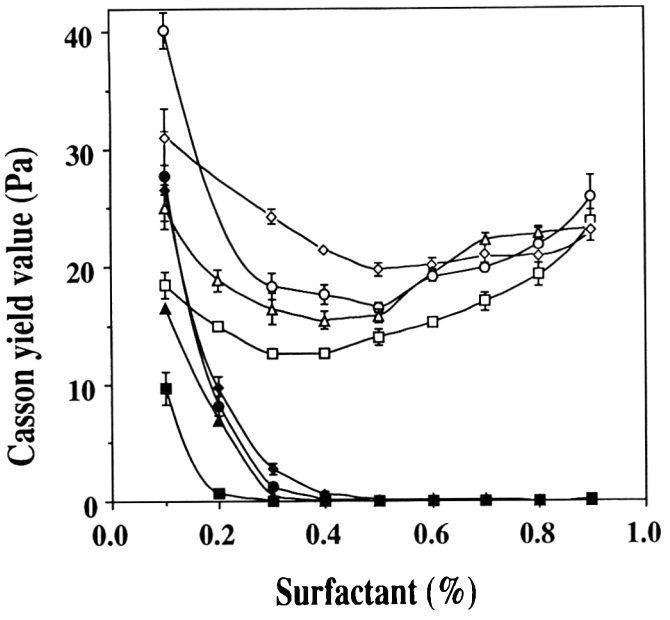


Fig. 2—Casson yield value of milk chocolate after conching relation to DWM and surfactant concentration: 0% lactose (lecithin: ◇, PGPR: ◆), 5% lactose (lecithin: ○, PGPR: ●), 12% lactose (lecithin: △, PGPR: ▲), 28% lactose (lecithin: □, PGPR: ■).

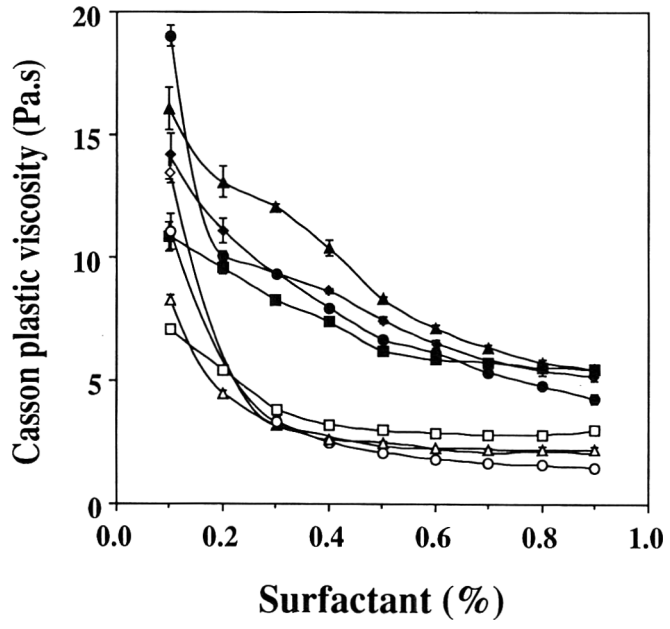


Fig. 3—Casson plastic viscosity of milk chocolate after conching relation to DWM and surfactant concentration: 0% lactose (lecithin: ◇, PGPR: ◆), 5% lactose (lecithin: ○, PGPR: ●), 12% lactose (lecithin: △, PGPR: ▲), 28% lactose (lecithin: □, PGPR: ■).

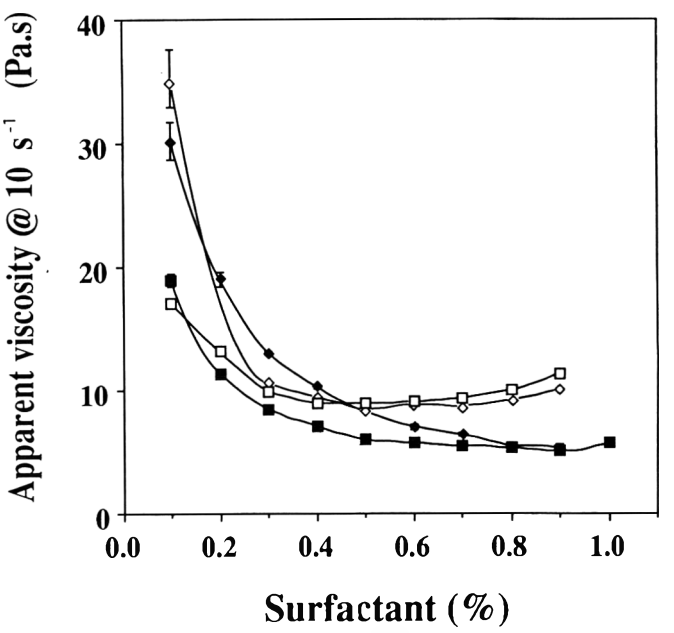


Fig. 4—Apparent viscosity @ 10 s^{-1} of milk chocolate after conching containing 0 (lecithin: ◇, PGPR: ◆) and 28% (lecithin: □, PGPR: ■) lactose from DWM as related to surfactant concentration.

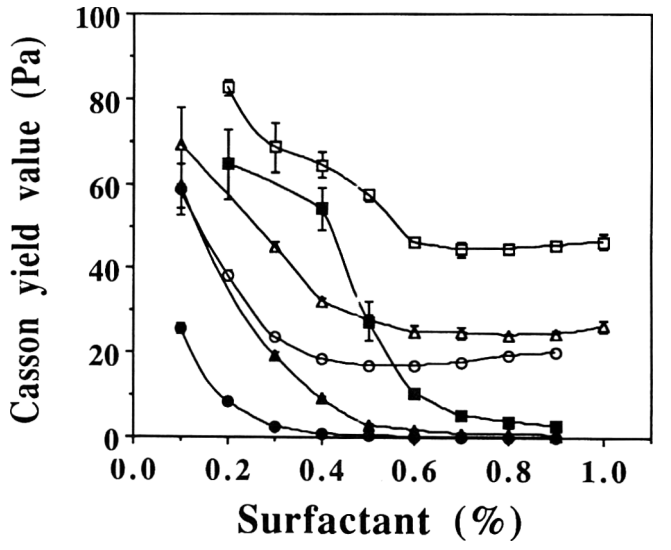


Fig. 5—Casson yield value of milk chocolate after conching as related to lactose from crystallized DWM and surfactant concentration: 5% lactose (lecithin: ○, PGPR: ●), 12% lactose (lecithin: △, PGPR: ▲), 28% lactose (lecithin □, PGPR: ■).

the remaining cocoa butter and 0.1% lecithin (Clearate B-70, W.A. Cleary Corporation, Somerset, NJ; single bleached, pourable, lecithin from Soya bean) were added at the beginning of conching. After 3 hr additional lecithin was added to obtain a final concentration of 0.3%. Low temperature conching for short times limits possible amorphous-crystalline transformations.

Chocolates were tempered immediately after conching using a Hilliard's Chocolate System (Jimsan Enterprises, Inc., West Bridgewater, MA) in a controlled temperature room at 16–18°C. The chocolate mass was brought to 48°C, held for 30 min, cooled to 28°C within 17 to 20 min, held for 10 min, heated to 30°C and held for 5 min. Tempered chocolate was poured into metal molds pre-heated to 30°C, shaken for 2 to 3 min on a vibrating bed (Syntron Jogger model J-1 B, FMC Corporation, Homer City, PA), and placed in a cooling cabinet at 15°C for 30 min. Proper demolding and good gloss were observed in all samples. The finished bars were wrapped in aluminum foil and stored at 20–22°C in plastic containers.

Analyses

Particle size distribution. Measurements of particle size distribution were made using the MasterSizer® laser diffraction particle size analyzer equipped with an MS 15 sample presentation unit and 100 mm lens (Malvern Instruments Ltd., Malvern, England). Refined milk chocolate was dispersed in isobutanol (Fisher Scientific, Pittsburgh, PA) (20–22°C) until an obscuration of 0.20 was obtained (0.016 to 0.030% volume concentration). Ultrasonic dispersion (50 W at 27 kHz) and mechanical stirring were applied for 5 min to assure that particles were independently dispersed.

Viscosity. The Casson model was used to evaluate the viscosity of milk chocolate (Office International du Cacao et du Chocolat, 1973). The equation relating the shear stress (τ) and the shear rate ($\dot{\gamma}$) at the

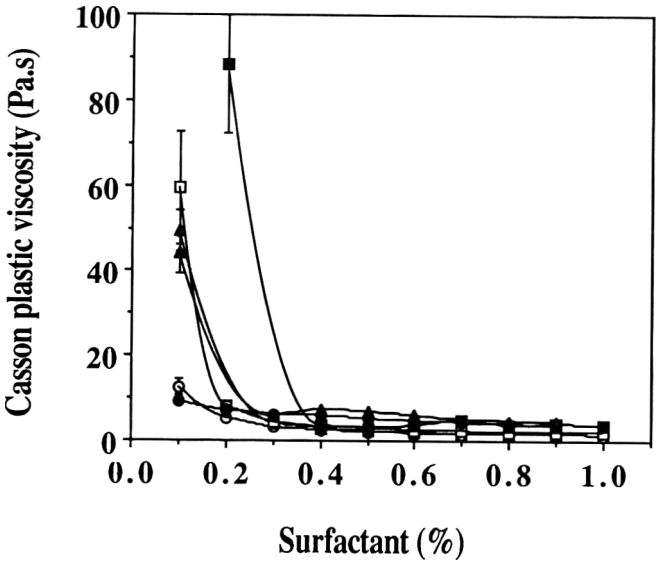


Fig. 6—Casson plastic viscosity of milk chocolate after conching as related to lactose from crystallized DWM and surfactant concentration: 5% lactose (lecithin: ○, PGPR: ●), 12% lactose (lecithin: △, PGPR: ▲), 28% lactose (lecithin: □, PGPR: ■).

surface of the inner cylinder for a Casson body in a concentric cylinder rotational viscometer follows:

$$(1 + a)\sqrt{\tau} = 2\sqrt{\tau_{CA}} + (1 + a)\sqrt{\eta_{CA}}\sqrt{\dot{\gamma}}$$

where a equals the ratio of radii of inner and outer cylinders, τ_{CA} is the Casson yield value, and η_{CA} the Casson plastic viscosity. The development of the I.O.C.C. method using the correction (1+a) was described by Steiner (1972).

Measurements were made at 40°C on samples taken at the end of conching using a rotational viscometer (Haake model RV12, Fisons Instruments, Valencia, CA) with concentric cylinder geometry, a=0.87 (model SVII, Fisons Instruments, Valencia, CA). Samples were transferred to the viscometer, left undisturbed for 10 min to stabilize the temperature, and then pre-sheared for 3 min at 17 s^{-1}. Shear stress was measured while increasing $\dot{\gamma}$ from 0–40 s^{-1} in 3 min, followed by decreasing $\dot{\gamma}$ from 40–0 s^{-1} in 3 min. Data from both ascending and descending ramps were used in calculation of the Casson parameters. All measurements were replicated using a second sample.

Interaction with surfactants. Casson parameters were used as an index of the influence of lactose on utilization of lecithin and polyglycerol polyricinoleate (PGPR, Palsgaard 4125, Emulsion Holland BV, Zierikzee, The Netherlands) at concentrations between 0.1 and 0.9%. The same milk chocolate recipes, refining and conching procedures were used, but the total batch size was 100g and final surfactant concentration 0.1%.

After conching, 30g of chocolate were transferred to a 100 mL sealed mixer (Omni-Mixer, Ivan Sorvall, Inc., Newton, CN) and homogenized (55°C) for 10 min at 1200 rpm. A 6 g sample was taken from the mixer and the viscosity measured as before, with the exception that τ was measured while increasing $\dot{\gamma}$ from 0–$\dot{\gamma}_{max}$ s^{-1} in 3 min, followed by decreasing $\dot{\gamma}$ from $\dot{\gamma}_{max}$–0 s^{-1} in 3 min, where $\dot{\gamma}_{max} \leq 40$. Slip was observed at $\dot{\gamma} < 40$ s^{-1} for a few of the more viscous samples, e.g., samples containing ≤0.2% surfactant. For those samples, $\dot{\gamma}_{max}$ was the maximum shear rate in the no-slip region, typically 10–30 s^{-1}. Samples were presheared at $0.43\dot{\gamma}_{max}$ if $\dot{\gamma}_{max}$ was ≤20 s^{-1}. The sample was returned to the mixer cup, and the total weight of chocolate in the mixer determined using an analytical balance (Model G200, Ohaus Scale Corporation, Florham Park, NJ). Surfactant was added to increase the concentration by 0.1% and mixing and viscosity measurements were repeated. Surfactant concentration was increased in 0.1% increments between 0.1 and 0.9%.

RESULTS & DISCUSSION

A FINELY GROUND DISCONTINUOUS PHASE, i.e., cocoa particles, sucrose crystals, and milk solids, must be present in milk chocolate to obtain the desired texture and mouthfeel. Particle size reduction is often achieved in 5-roll refiners by the pressure and shear forces of the rotating rolls in the roll gaps. CDWM with 70% nominal lactose produced chocolates which required longer times for the first pass through the refiner due to the presence of aggregates between 2 and 3 mm in size. No differences were observed during the second pass.

Differences in dispersed phase particle size distribution were observed with varying lactose content and milk powder type. Unimodal volume-based particle size distributions were typical (Fig. 1A). More fine particles, 0.2-3 μm, were observed in chocolate containing 28% lactose from CDWM (Fig. 1B). These particle size distributions were consistently observed in replicate samples.

Table 2 shows the median, volume-based diameter, d[v, 0.5], of the prepared milk chocolates. The lowest d[v, 0.5] was observed for chocolates containing 12 or 28% lactose from CDWM. The largest d[v, 0.5] was observed in milk chocolate containing 28% lactose from DWM. Particle size increased in chocolates containing DWM as the lactose concentration increased from 5 to 28%. Chocolate containing 0% lactose did not follow this trend, probably as a result of lower milk powder particle density as compared to DWM containing lactose (Aguilar and Ziegler, 1993b).

Post-drying crystallization resulted in accretion of particles (Aguilar and Ziegler, 1993b). Examination by scanning electron microscopy revealed that these large aggregates comprised many small lactose crystals (Aguilar and Ziegler, 1993b). Refining may have simply separated these small crystals, resulting in the high percentage of fines in chocolate with CDWM (Fig. 1B).

Particle size reduction during chocolate refining results from compression between the rotating rolls and the crushing of softer particles between harder particles. DWM prepared by the method of Aguilar and Ziegler (1993b) had a greater vacuole volume (34–53 mL/100 g) than CDWM (3.8-34 mL/100g). Air vacuoles in milk particles may determine the particle fracture pattern during refining and, therefore, particle size distribution in refined chocolate.

Lactose content and milk powder type had a significant effect on the flow behavior of molten, conched milk chocolate. That containing 28% lactose from CDWM exhibited the highest Casson yield value, τ_{CA} (Table 3). Chocolates containing 28% lactose from DWM had the lowest τ_{CA}. The τ_{CA} of chocolates containing DWM decreased as lactose concentration increased. This was consistent with reports that τ_{CA} is inversely related to particle size (Chevalley, 1994). The τ_{CA} of chocolates with CDWM (i.e., crystalline lactose) increased sharply at 28% lactose (Table 3), though median particle size was unchanged (Table 2). This was most likely due to the presence of additional fines (Fig. 1B). Values of η_{CA} and η_{app} @ 10 s^{-1} paralleled the trend in τ_{CA} (Table 3). These observations were consistent with Hogenbirk (1985).

Free fat in dry whole-milk powder becomes part of the continuous fat phase reducing chocolate viscosity during conching (Verhey, 1986). A decrease in viscosity was expected as a result of increased free fat in CDWM (Aguilar and Ziegler, 1993b). This anticipated viscosity reduction may have been obscured by a viscosity increase due to particle size distribution.

The density of DWM increased with increasing lactose concentration (Aguilar and Ziegler, 1993b). The viscosity of a particulate suspension increases with the volume fraction of the dispersed phase. For a given weight % in a chocolate formulation, particles with higher density occupy smaller volume and, therefore, the viscosity of the suspension would be lower. That is, for the same viscosity less cocoa butter would be required to suspend the particles. The effect of vacuole volume (density) on milk chocolate viscosity was investigated by Dodson et al. (1984), who confirmed this by demonstrating that large vacuole volume resulted in high chocolate viscosity.

A minimum τ_{CA} was observed at lecithin concentrations between 0.3-0.5% for chocolates with DWM (Fig. 2). At lecithin concentrations higher than 0.5% τ_{CA} increased. τ_{CA} was almost negligible at polyglycerol polyricinoleate (PGPR) concentrations

between 0.3–0.5% (Fig. 2). These results were consistent with common experience (Seguine, 1993). The minimum τ_{CA} occurred at lower concentrations of lecithin or PGPR as the concentration of lactose from DWM increased. Lecithin was more effective at reducing η_{CA} than PGPR (Fig. 3).

Both η_{CA} and τ_{CA} affect the apparent viscosity, η_{app}. PGPR had a greater influence on τ_{CA}, while lecithin had a stronger influence on η_{CA}. The net result was that the two surfactants had a similar effect on η_{app} (Fig. 4).

τ_{CA} was followed as a function of lactose and surfactant concentration for chocolates containing CDWM (Fig. 5). As compared to lecithin, lower τ_{CA} were obtained with PGPR at the same lactose content. Contrary to results with DWM, τ_{CA} increased with concentration of CDWM. PGPR was effective in reducing τ_{CA}; however, more PGPR was needed with CDWM as compared with DWM.

At low surfactant concentrations, lecithin was more effective than PGPR for reducing η_{CA} of milk chocolates with 28% lactose from CDWM (Fig. 6). Increasing the concentration of lactose from CDWM resulted in higher η_{CA} at low surfactant concentrations. The increase in fine particles in chocolate with increasing amounts of CDWM would result in a greater specific surface area, and a greater requirement for surfactant.

CONCLUSIONS

THE CONCENTRATION AND PHYSICAL FORM of lactose in dry whole milk used in the manufacture of milk chocolate influenced the dispersed phase particle size distribution, Casson yield value, plastic viscosity, and the requirement for surfactants. Milk chocolate with increased amorphous lactose from DWM prepared by conventional spray-drying had larger post-refining particle size, reduced viscosity, and a lower requirement for surfactant. Milk chocolate with increased crystalline lactose from DWM prepared by post-drying crystallization had smaller post-refining particle sizes, higher viscosity, and greater requirement for surfactants. Storage of commercially spray-dried DWM (with amorphous lactose) under adverse conditions, e.g., high temperature or high relative humidity, resulting in lactose crystallization could affect flow properties of chocolate containing it.

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Partitioning of Printing Ink Solvents on Chocolate

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ABSTRACT

Partitioning (Kp) of printing ink solvents was studied in three types of chocolate with different fat content and degrees of crystallinity. At 25°C, chocolate liquor (48% fat and medium crystallinity) showed a higher Kp than chips (25% fat and higher crystallinity), but cream (29% fat and lowest crystallinity) showed the highest Kp. Order appeared to depend on degree of crystallinity. At 35°C and 45°C as samples became increasingly molten, effect of crystallinity diminished and order of increasing Kp depended on fat content. Kp order of solvents was the same in all chocolates and temperatures: toluene > isopropanol > methyl ethyl ketone > ethyl acetate > hexane in accord with Hildebrand solubility parameter differences.

Key Words: chocolate, ink solvents, solvent partioning

INTRODUCTION

ON FOOD PACKAGES printing ink solvents, consisting of various aliphatic and aromatic compounds, may sometimes cause off-flavors in food by migration of residual solvent from the package (Gilbert, 1976). Studies have been conducted on off-flavors due to migration of solvents in packaging materials (Piringer, 1986; McGorrin et al., 1987) and some studies have measured partition coefficients between solvents and food samples (Heydanek et al., 1979; Halek and Hatzidimitriu, 1988). Very few publications have reported factors that affected the partitioning between solvents and solid food samples (Halek and Levinson, 1988, 1989; Halek and Chan, 1994). Data are sparse on the relation of food composition and properties to such partioning.

Our objective was to determine the effects of fat content and structure in chocolate on partition coefficient (Kp) values using thermodynamic values to interpret observed migrations. We determined fat content, individual fatty acid components, and melting range of three types of chocolate samples. They were selected (chips, liquor, and cream) to represent different fat content and different degrees of crystalline structure. Five printing ink solvents were chosen to represent different specific functional groups. Three temperatures selected were below the melting point (25°C), very close to the melting point (35°C), and above the melting point (45°C).

MATERIALS & METHODS

Solvents

Hexane, toluene, isopropanol, methyl ethyl ketone, and ethyl acetate of 99.9% purity were obtained from Fisher Scientific Co.. Reagents to analyze fatty acid composition were: sodium hydroxide, methanolic solution of BF_3 (12.5% BF_3 content and 99.9% purity), hexane (99.9% purity), and anhydrous sodium sulfate, all from Fisher Co.. Saturated sodium chloride was obtained from Aldrich Co..

Chocolate

Three chocolates were obtained from a local food company and tightly wrapped in aluminum foil and stored in a refrigerator at 3°C. They represented a range of chocolates with different fat and melting characteristics employed in making cookies. Our analysis showed the following characteristics, Chips (25.4% fat, melting point range, 35.5–37°C), Liquor (48.4% fat, 35–36.5°C) and Cream (28.9% fat, 34–35°C). Composition of fatty acids in the fats was determined (Table 1).

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Table 1—Analysis of fat components

Sample (% fat)	Fatty acids in triglycerides	Mass ratio	Ratio (Stearic and Palmitic acid)/(Oleic and Linoleic acid)[a]
Chips (25.4)	Stearic, Palmitic, Oleic and Linoleic acid[a]	4.8:3.3:3.6:1.0	1.76:1.00
Liquor (48.4)	Stearic, Palmitic, Oleic and Linoleic acid	4.6:2.9:4.1:1.0	1.47:1.00
Cream (28.9)	Stearic, Palmitic, Oleic and Linoleic acid	4.1:2.8:4.5:1.0	1.25:1.00

[a] Stearic acid (mp: 69.6°C) Tristearin (mp: 72.5°C)
Palmitic acid (mp: 62.9°C) Tripalmitin (mp: 66°C)
Oleic acid (mp: 13.4°C) Triolein (mp: 5.5°C)
Linoleic acid (mp: −5.0°C) Trilinolein (mp: −13°C)

Table 2—Melting ranges of samples

Sample	Starting point	Ending point	Melting time[a]
Chips	35.5°C	37°C	12 min
Liquor	35°C	36.5°C	10 min
Cream	34°C	35°C	4 min

[a] Heating rate of 0.2°C/min.

Fat content and fat components

The modified method of Paquot and Hautfenne (1987) was used. Duplicate samples (5.0 g) were extracted in a Soxhlet apparatus with 150 mL hexane for 7 hr. Hexane was removed by distilling into the extraction apparatus, and the concentrated solution was transferred to a tared 100 mL flask to complete removal. The flask was cooled in a desiccator and weighed for determining fat weight. The fatty material was converted to esters by boiling with 7 mL methanolic NaOH solution followed by 10 mL methanolic solution of BF_3. 10 mL hexane was then added and saturated sodium chloride solution was added to the neck of the flask (100 mL). The mixture was separated in a separatory funnel (100 mL) and the upper level was dried with anhydrous sodium sulfate (0.3–0.5g). 1 µL was injected into a Varian 3300 Gas Chromatograph with flame ionization detector and silica capillary column, [Supelcowax 10 (0.32 mm × 30 m) from Supelco Co.]. Operating conditions included: He carrier gas at 1.5 cc/min. and injection port 230°C, column 145°C, and detector 230°C. A Varian 4270 recorder-integrator was used. Fatty acid components were identified and determined by comparing with authentic standard fatty acid methyl ester peak retention times and peak areas.

Melting range of chocolate samples

Melting range was determined on 0.3g of each sample between cover glasses on a thermal hot stage under a Bristoline thermal microscope with polarizer/analyzer and thermometer. The temperatures at which the first and last crystals disappeared at a heating rate of 0.2°C/min were recorded.

Partition coefficients

Partition coefficient values were determined in a closed system by a method described previously (Biran et al., 1979). 70 mL bottles with individual chocolate samples weighed (± 1mg) and blank bottles were

Table 3—Average Kp for each solvent at 25, 35, and 45°C

Sample	Solvent	25°C (± STD[a])	35°C (± STD[b])	45°C (± STD[b])
Chips	Toluene	0.0667 ± 0.0030	0.0942 ± 0.0011	0.1073 ± 0.0017
	Isopropanol	0.0615 ± 0.0030	0.0800 ± 0.0018	0.0907 ± 0.0013
	MEK	0.0410 ± 0.0014	0.0722 ± 0.0019	0.0831 ± 0.0014
	Ethyl acetate	0.0335 ± 0.0024	0.0464 ± 0.0003	0.0521 ± 0.0004
	Hexane	0.0251 ± 0.0021	0.0411 ± 0.0003	0.0459 ± 0.0003
Liquor	Toluene	0.1063 ± 0.0037	0.1832 ± 0.0064	0.2207 ± 0.0031
	Isopropanol	0.0973 ± 0.0025	0.1595 ± 0.0024	0.1696 ± 0.0021
	MEK	0.0764 ± 0.0046	0.0938 ± 0.0006	0.1115 ± 0.0010
	Ethyl acetate	0.0441 ± 0.0028	0.0685 ± 0.0003	0.0802 ± 0.0023
	Hexane	0.0342 ± 0.0011	0.0597 ± 0.0008	0.0708 ± 0.0022
Cream	Toluene	0.1699 ± 0.0032	0.1758 ± 0.0010	0.1749 ± 0.0037
	Isopropanol	0.1029 ± 0.0073	0.1022 ± 0.0022	0.1094 ± 0.0008
	MEK	0.0786 ± 0.0061	0.0859 ± 0.0016	0.0865 ± 0.0008
	Ethyl acetate	0.0566 ± 0.0011	0.0577 ± 0.0006	0.0594 ± 0.0022
	Hexane	0.0500 ± 0.0021	0.0550 ± 0.0010	0.0537 ± 0.0016

[a] Average of determinations on three samples at four concentrations over 10–150 ppm, N = 12.
[b] Average of determinations on three samples at one concentration over 50–70 ppm, N = 3.

Table 4—Hildebrand solubility parameter value of each solvent

Solvent	δ ($MPa^{1/2}$)
Isopropanol	21.0
Toluene	18.7
Methyl ethyl ketone	18.4
Ethyl acetate	17.9
Hexane	14.9
Triglyceride	18.6

prepared and sealed with septa. For 25°C determinations, bottles were injected with 0.1, 0.2, 0.4, 0.8 µL of each solvent. For 35°C and 45°C determinations, all bottles were injected with 0.4 µL. Volumes were converted to mass for Kp determination from densities of the pure solvents. Equilibrium times for each solvent at each temperature were determined by plotting % difference between control and sample headspace values until they did not change. Equilibrium time varied from 1 to 3 days according to solvent and storage temperature. After storing each bottle for 1 day more than the equilibrium time at the specified temperature, a 200 µL sample of headspace was injected into the GC by gastight syringe. A Varian 3300 Gas Chromatograph with flame ionization detector and silica capillary column, DB-1 (0.25 mm × 30m) from Supelco Co. were used. Additional operating conditions included He carrier gas at 1 mL/min. and injection port 230°C. column 70°C and detector 230°C. A Varian 4270 recorder-integrator was used. The amount of solvent absorbed by the sample was determined by the difference between amounts in the headspace in the sample and blank bottle. These amounts were determined from a calibration chart of G.C. areas vs. µg of pure solvent injected into the G.C.. These experiments were repeated 3 times. Partition coefficients between chocolate samples and headspace were calculated by the following equation (Halek and Levinson, 1988):

$$Kp = \frac{\left(\begin{array}{c}\text{wt of solvent in}\\ \text{headspace in blank}\end{array}\right) - \left(\begin{array}{c}\text{wt of solvent in headspace}\\ \text{in bottle with sample}\end{array}\right)}{\dfrac{\text{wt of sample}}{\dfrac{\text{wt of solvent in 1 mL headspace in bottle with sample}}{\text{density of air}}}}$$

RESULTS & DISCUSSION

Fat composition and effect of temperature on crystalline state

The relative effects on Kp of differences in % fat content and degree of crystalline structure of chocolates were determined. Each chocolate fat sample had different ratios of fatty acids in their various triglycerides (for example, palmitins, stearins, etc.). Among the fat components, the decreasing order of melting point was tristearin (72.5°C) > tripalmitin (66°C) > triolein (5.5°C) > trilinolein (−13°C). These values were close to those of the respective fatty acids. The ratios of each fatty acid component in the triglycerides were compared (Table 1) as well as the melting point range of each chocolate sample (Table 2). Chips had the highest relative amounts of high melting stearin and palmitin, and had the highest and longest melting range. Chips chocolate was the most crystalline at 25°C and 35°C, and was molten at 45°C. Chocolate liquor was also crystalline at 25°C but became partially molten at 35°C. The cream was the least crystalline at 25°C and melted rapidly as expected from its composition.

Partition coefficient variations with temperatures

Kps for each solvent at 25, 35, and 45°C (Table 3) increased with temperature. At 25°C the order of Kp values was : cream > liquor > chips, which was the order of increasing fat crystallinity, but not the order of fat content. While the liquor sample (48% fat content) showed a higher Kp value than chips (25% fat content), the cream sample (29% fat content) showed an even higher Kp value than the liquor sample although it had lower fat content. At 25°C, degree of crystallinity in structure was more important than fat content. Previous work with these printing ink solvents in soybean oil, chocolate liquor and high fat cookies (Halek and Hatzidimitriu, 1988) had shown an increase in Kp fat/air with an increase in fat content and some dependency on solvent concentration at low concentrations of solvent vapors. The possibility of a concentration dependency was examined to eliminate it as a source of variability in Kp determinations. In the concentration range (10 - 150 ppm) of each solvent in chocolate/air systems, Kp values were not dependent on solvent concentration. Partition coefficient values at 35 and 45°C showed a change from the order found at 25°C. At 35°C, the order was liquor (48.4% fat) > cream (28.9% fat) > chips (25.4% fat). Thus, as structures of chocolates became less crystalline, fat content affected the partition coefficient values to a greater degree. At 45°C, the structure in each sample became more amorphous and the order of Kp value was the same as that at 35°C, but Kp differences were increased.

Solvent affinity order

The order of Kp values for each solvent in each chocolate was the same at all temperatures: toluene > isopropanol > methyl ethyl ketone > ethyl acetate > hexane. This may be explained by comparing Hildebrand solubility parameter (δ) values for the solvents and triglycerides (Barton, 1991) (Table 4). The Hildebrand equation for heat of mixing shown below, predicts that compounds with similar δ have a greater tendency to mix (Hildebrand et al., 1970).

$$\Delta Hmix = V_1(\delta_1 - \delta_2)^2 \emptyset_2 \qquad (1)$$

where ΔHmix is the partial molar heat of mixing; V_1 is the partial molar volume of the penetrant; $\emptyset_2$ is the volume fraction of the absorbent in the mixture; δ_1 and δ_2 are the square roots of the cohesive energy density of the absorbent and penetrant.

Table 5—ΔH (enthalpy), ΔG (free energy), and ΔS (entropy) values of each solvent[a]

Sample	Solvent	Δ (cal/mol) 25°C	Δ (cal/mol) 35°C	Δ (cal/mol) 45°C	ΔS (cal/mol*K) 25°C	ΔS (cal/mol*K) 35°C	ΔS (cal/mol*K) 45°C	ΔH (cal/mol)
Chips	Toluene	1606	1446	1411	9.17	9.40	9.21	4340
	Isopropanol	1665	1546	1517	6.91	7.07	6.94	3723
	MEK	1918	1609	1572	16.31	16.79	16.37	6779
	Ethyl acetate	1973	1880	1867	4.84	4.98	4.86	3414
	Hexane	2133	1954	1948	8.58	8.88	8.62	4690
Liquor	Toluene	1327	1039	955	17.68	18.04	17.74	6596
	Isopropanol	1374	1123	1121	11.95	12.38	12.00	4936
	MEK	1516	1448	1386	6.02	6.05	6.05	3310
	Ethyl acetate	1804	1641	1594	9.81	10.02	9.85	4727
	Hexane	2006	1725	1674	15.69	16.10	15.75	6683
Cream	Toluene	1065	1064	1084	−1.94	−1.87	−1.87	488
	Isopropanol	1332	1396	1398	−3.38	−3.48	−3.37	325
	MEK	1474	1502	1547	−3.68	−3.66	−3.68	376
	Ethyl acetate	1694	1746	1784	−4.56	−4.58	−4.56	334
	Hexane	1744	1775	1847	−5.20	−5.13	−5.19	195

[a] Determination at concentration over 50 ppm - 70 ppm.

Using Eq. [1] and the δ values (Table 4) yields the following increasing order of ΔHmix values in J/mol: toluene 0.5, MEK 5, ethyl acetate 52, isopropanol 439 and hexane 1811. Thus toluene would mix more easily than hexane, for example, and the data predicts the series that was found experimentally except for isopropanol. The isopropanol anomaly may be due to the high polarity of its-OH functional group that can participate in hydrogen bonding in the chocolate compositions and predominate over the order of attractive forces indicated by its δ value, which is influenced only by dispersion forces.

Thermodynamic values

From the Kp data at 25 to 45°C (Table 3) Arrhenius plots of ln Kp vs 1/T produced enthalpy values (ΔH) for the sorptions from Eq. [2] (Table 5).

$$\ln Kp = 1/T*(-\Delta H/R) \quad (2)$$

Here R = Gas constant (1.98717 cal/mol*K); T = Absolute temperature (°K).

Gibbs free energy values (ΔG) produced from Eq. [3] are listed in Table 5.

$$\Delta G = -RT \ln Kp \quad (3)$$

Finally, entropy (ΔS) values were calculated from Eq. [4].

$$\Delta S = (\Delta H - \Delta G)/T \quad (4)$$

Among the three chocolates, the decreasing order of ΔG was chips > liquor > cream at 25°C, but at 35 and 45°C the order changed to chips > cream > liquor. This indicated that melting of chips and liquor at higher temperatures increased the free volume available for solute and raised their partition coefficient. The amorphous cream did not show this effect. This was further supported by the ΔH from the Arrhenius plots that showed low values for cream and higher for chips and liquor. Apparently the calculated values for ΔH were confounded with the ΔH due to different degrees of fusion of chocolates with temperature. That is reflected by the large differences in ΔH for chips and liquor compared to cream. That has further affected the calculated values for ΔS which depended on ΔG and ΔH. In addition, factors other than fusion of chocolates, such as positive contributions of heat of vaporization of the solvent and negative contribution from $V_1 (\delta_1 - \delta_2)^2$ are included in the table ΔH calculated from Arrhenius plots. Qualitatively the entropy values for the cream were lower than those of the chips and liquor, which indicated little additional disorder in the cream compared to larger changes possible in the chips and especially in the high fat content liquor.

CONCLUSIONS

CHANGES OF STATE OF INGREDIENTS in a solid food with temperature can change the partitioning degree for volatile compounds into the food. These changes occurred in a temperature range commonly found during shipping and storage of unrefrigerated packaged food. Thus, data at only one temperature are not enough to permit estimating amounts of volatiles transferring into foods. This is particularly important when the volatiles can cause off-flavors at low concentrations and must be known for the conditions of use.

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Heating Cruciferous Vegetables Increases *in Vitro* Dialyzability of Intrinsic and Extrinsic Iron

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ABSTRACT

Iron dialyzability (ID) from three *Cruciferae* (broccoli, kale and cabbage) was determined by an *in vitro* digestion method. The effect of added Crucifers on the ID of extrinsic nonheme iron as well as effects of heating were studied. Uncooked Crucifers contained iron of moderate ID (7–9%); cooking resulted in substantial ID increase (200%). Cooked Crucifers increased extrinsic ID three- to fourfold. Time and temperature relationships for the increase suggested that organic acids released after partial cell wall degradation combined with protein denaturation and iron solubilization from fiber were major reasons for differences between raw and cooked vegetables. Cruciferous vegetables can contribute to improved iron nutrition.

Key Words: iron, *in vitro* dialyzability, bioavailability, *Cruciferae*, fiber

INTRODUCTION

IRON DEFICIENCY ANEMIA, the most prevalent nutritional problem affects 500–600 million people worldwide (Baynes and Bothwell, 1990). Diet composition is critical since low iron intake and/or bioavailability (IB) are main causes of the deficiency (Fairweather-Tait, 1984). Iron contributes to two pools in the body, for heme iron (HI) and nonheme iron (NHI) which account for 10 and 90% of dietary iron respectively. HI absorption is high and diet-independent (Sweeten et al., 1986); NHI absorption, although variable, is considered low and greatly influenced by interactions with enhancers and inhibitors (Layrisse et al., 1969; Cook, 1983). Meat (Monsen et al., 1978), ascorbate (Monsen, 1982) and citrate (Gillooly et al., 1983) increases IB whereas fiber (Reinhold, 1982), tannins (Disler et al., 1975), phytate (Torre et al., 1991) and certain proteins (Kratzer and Vohra, 1986) act as inhibitors.

Iron from different foods exhibits variable IB. Meat is a significant iron source as it contains bioavailable HI. However, NHI found in cereals, nuts and legumes exhibits low IB due to high concentrations of inhibitors (Hazell and Johnson, 1986). High variability has been observed among vegetables (Gillooly et al., 1983). Some, such as spinach (Gordon and Chao, 1984), provide poor sources of iron and only speculations for significant IB exist for others. The *Cruciferae* family includes vegetables such as broccoli, cabbage and several green leafy vegetables. They contain high amounts of important nutrients and anti-cancer agents which have brought about increased interest in them (Caragay, 1992). The presence of iron and organic acids in Crucifers indicates they may serve both as a source of bioavailable NHI and an enhancer of NHI absorption from other foods. Sharp IB changes may occur after processing, since it affects the food matrix and alters the iron chemistry (Lee, 1982) making processing optimization necessary for iron retention and IB. No systematic *in vitro* study of IB has been reported on Crucifers.

We used the Miller et al. (1981) method to estimate *in vitro* iron dialyzability (ID). Schricker et al. (1981) have shown that this fast and low cost method correlated highly with *in vivo* data on identical meals and that ID could provide a reliable index of IB. Our objectives were (a) to estimate the *in vitro* ID of three raw and cooked Crucifers, (b) to study the effects of their addition on the *in vitro* ID of extrinsic iron, and (c) to evaluate the effects of cooking on the ID of Crucifers.

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MATERIALS & METHODS

Glassware and reagents

All glassware used was washed with distilled water, left overnight in 1M sulfuric acid bath to remove possible iron contamination and rinsed several times with distilled deionized water.

Pepsin solution. 16g of pepsin 1:10,000 powder from hog stomach (Sigma) were dissolved in 100 mL 0.1 M HCl (trace metal grade, Fisher). The solution was prepared fresh weekly and kept at 4°C.

Pancreatin-bile (PB) suspension. 1 g pancreatin from porcine pancreas and 6.5g porcine bile extract (both from Sigma) were suspended in 250 mL of 0.1 M $NaHCO_3$. The suspension was prepared fresh weekly and stored at 4°C.

Iron stock solution. 1015 ppm Fe^{3+} stock solution (Aldrich, Milwaukee, WI) in 0.1N HCl was used for preparation of AAS standard solutions.

Preparation of vegetables

Broccoli (*Brassica oleoracea L.* var. italica Plenk) kale (*Brassica oleoracea L.* var. acephala D.C.) and cabbage (*Brassica oleoracea L.* var. capitata) were studied (average 1.3, 1.9 and 0.5 ppm Fe respectively). The vegetables were purchased from a local supermarket (New Brunswick, NJ) and analyzed immediately or after brief storage. We washed, air-dried and cut the vegetables, removing inedible parts. We cooked the vegetables by boiling cut leaves or florets in a covered glass beaker containing 100–200 mL distilled deionized water (depending on water content of vegetable). Use of glass exclused iron leaching experienced with metallic utensils (Mistry et al., 1988). Boiling time was 15 min, measured after adding the vegetables in boiling water. At the end of treatment, the beaker and its contents were quickly cooled to room temperature (≈25°C) using running tap water (to avoid changes due to elevated temperature after 15 min). We retained the boiling water as it contained appreciable soluble (and thus highly bioavailable) iron (Gillooly et al., 1983; Latunde-Dada, 1990) and used it for preparation of initial homogenates. Microwave cooking was performed in a 600 W microwave oven with rotating base (81-MDS, CEM Corp.). Broccoli was heated for various times in a covered glass beaker containing 50 mL water. ID values of raw and cooked vegetables were determined the same day. The ID values for each vegetable are averages of triplicate batches with three values for each batch.

Iron determination in raw vegetables

We dried the vegetables (AOAC, 1990) to avoid errors caused by water evaporation. Dried samples were ground and 0.5 g was weighed in Teflon digestion vessels (CEM Corp.). We added 10 mL N HNO_3 and after 30 min, we added 5 mL of 10 N HCl. After gas production subsided, we capped and placed the vessels in the microwave digestion oven turntable (MDS-81D, CEM Corp.) The samples were digested using 100% power for 5 min and 70% for 8 min (internal pressure monitored by CEM pressure controller). We diluted the digests to 25 mL, filtered and transferred them in vials. Iron concentration of the digests was determined by Atomic Absorption Spectrophotometer (AAS, model 603, Perkin-Elmer Corp.) with air-acetylene flame at 248.3 nm (slit length 2 nm). The calibration curve was established using standards of 0.1, 0.3, 0.5, 1, 2, 4, 5, 8 and 10 ppm Fe. The relationship between iron concentration and absorbance was linear with high correlation coefficients (R^2>0.99).

In vitro determination of iron dialyzability

The simulated digestion method of Miller et al. (1981) was used with minor modifications. Vegetable sample (150–200 g) was blended in a food processor (Cuissinart, Norwich, CT) with appropriate water (100–200 g) to provide a mixture with creamy consistency and 5–7% dry matter. The pH of the mix was adjusted to 2.0 ± 0.05 by addition of 6N HCl. Four aliquots of 20 g were taken in 125 mL flasks, 0.75 mL pepsin solution was added, the flasks were covered, locked and incubated for 2 hr at 37°C. After pepsin digestion, the titratable acidity (Miller et al. 1981) was determined in one of the aliquots. We used pH = 7.00 ± 0.05 as the titration stop point (instead of 7.5). The final pH of the dialyzates and retentates using that value was 6.0–6.4, nearer the pH of human duodenum. This better represented the biological process and increased the levels of dialyzable iron to values similar to those from *in vivo* studies (Whittaker et al., 1989). We then placed 25 mL of a solution containing an amount of $NaHCO_3$ equivalent to the titrable acidity, in presoaked dialysis tubing (6000–8000 m.w. cut-off number), submerged the tubing in the digests and incubated them for 30 min at 37°C. We added 5 mL of PB suspension and continued the incubation for 2 hr. After removing and rinsing the tubings, we transferred and weighed their contents. Iron concentration in the dialyzates was determined by the AAS procedure used for microwave digests of raw vegetables. AAS was preferred over bathophenanthroline method ("batho") based on preliminary experiments (Kapanidis, 1993).

The equation for calculation of ID was:

$$\text{Iron dialyzability (\%)} = \frac{[Fe]_{dial} * wgt_{dial}}{[Fe]_{veg} * wgt_{veg}/(wgt_{veg} + wgt_{water})}$$

where $[Fe]_{dial}$ = iron in dialyzates (μg/g); $[Fe]_{veg}$ = iron in vegetables, wet basis (μg/g); wgt_{dial} = dialyzates after dialysis (g); wgt_{veg} and wgt_{water} vegetables and water used in preparation of initial homogenate (g).

Soluble iron (SI) determinations

We determined the concentration of soluble iron in various digestion stages by AAS. This was done after centrifugation of 25–30 mL aliquots (45,000 g, 10°C, 20 min) in a refrigerated centrifuge (Superseed RC2-B, Sorvall) and filtration through 0.45 μm filters.

RESULTS & DISCUSSION

ID of raw and cooked Crucifers

The *in vitro* ID of raw and cooked broccoli, kale and cabbage was determined (Table 1a). Boiling resulted in an increase ($P<0.005$) of ID (202% increase for broccoli, 207% for kale and 200% for cabbage). This suggested that heating could alter the iron profile and influence the ID and IB (Hazell and Johnson, 1987). This increase reinforces the possible unreliability of basing IB results entirely on the composition of raw foods. That would not consider any physicochemical changes in food components which may be caused by processing (Lee, 1982).

After comparing the ID to those of similar *in vitro* studies on plant and animal foods (Hazell and Johnson, 1986, 1987; Latunde-Dada, 1990), raw Crucifers seemed to contain iron of moderate ID (7–9%). Iron in cooked Crucifers was much more dialyzable (22–29%). Such high ID is mainly a result of high iron solubility at neutral pH and in the intestines. High solubility is necessary for iron absorption. This is achieved through two mechanisms. The first is strong chelation and complexation (especially of ferric iron) by compounds with good electron donors. Second is iron reduction to more soluble (at neutral pH) ferrous form (Clydesdale, 1983). Ascorbate contributes to both mechanisms, with chelating ability more pronounced at neutral pH (Clydesdale, 1982; Gorman and Clydesdale, 1984). Although sometimes lost during cooking, the conditions we used (cover, small amounts of water, water retained) kept most of the ascorbic acid in active form (Salunkhe et al., 1974). Furthermore, short-chain organic acids, such as citric and malic acid, are effective iron-chelating agents, and form stable iron complexes (Rao and Rao, 1985). Such acids can also chelate iron which has been complexed in insoluble compounds (e.g. fiber and phytate complexes), thus solubilizing the iron. We hypothesize that ID increase was related to the high concentration of ascorbic, acid and malic acid in Crucifers and their release after heating (Souci et al., 1989).

Table 1—*In vitro* ID of three Crucifers before and after boiling 15 min and *in vitro* ID of controls (17.5 ppm Fe as $FeCl_3$) compared to ID of raw and cooked vegetables containing same amounts of extrinsic iron

	Raw	Cooked
Intrinsic iron		
Broccoli	7.52 ± 0.50	23.11 ± 1.47
Kale	7.44 ± 0.74	22.44 ± 1.20
Cabbage	9.70 ± 1.64	29.14 ± 1.42
Extrinsic iron		
Control	7.60 ± 0.27	
Broccoli	7.94 ± 0.73	28.67 ± 2.02
Kale	11.25 ± 0.10	31.09 ± 1.28
Cabbage	3.22 ± 0.17	38.21 ± 0.70

Effect of Crucifers on the ID of extrinsic iron

The effect of addition of raw and cooked kale, broccoli and cabbage on the ID of a sample containing a standard amount of added iron was determined (Table 1b). A volume of $FeCl_3$ solution (1015 μg Fe/mL) was added in the mixture prior to pepsin digestion so that the extrinsic iron concentration in the homogenate was 17.5 ppm (Kane and Miller, 1984). $FeCl_3$ was used because it is the most common source of extrinsic iron for *in vivo* experiments, successfully simulating NHI. A blank sample containing $FeCl_3$ and no vegetable was the control.

As with the first experiment, cooked vegetables increased ($P<0.005$) the ID of control iron solution (277% for broccoli, 308% for kale and 403% for cabbage). Raw vegetables exhibited different effects: broccoli did not alter the ID of $FeCl_3$, cabbage depressed it (58% decrease, $P<0.005$) and kale increased it (48%, $P<0.001$). The similarity of trends between results of our first 2 experiments implied that plant interactions with both intrinsic and extrinsic iron were similar; the same enhancers and inhibitors were probably involved. Additionally, the high ID of intrinsic iron in the cooked plant could not be solely attributed to iron liberation from a low IB form. Rather, the generation of a strong enhancer or deactivation of an effective inhibitor of iron absorption (that could equally affect the ID of extrinsic iron) is far more likely. Hence, extrinsic iron probably interacted with a plant constituent that was substantially altered during cooking. Such constituents could be dietary fiber, proteins and polyphenols (potential inhibitors) and ascorbate, citrate, malate, sugars and amino acids (cysteine, histidine and glycine) or peptides generated by heat and enzymatic proteolysis (potential enhancers).

Effect of boiling time

The effect of boiling time on the ID of broccoli florettes was determined (Fig. 1). When Fig. 1 florettes were boiled, ID increased rapidly and then stayed constant for up to 30 min with a slight increasing tendency. The generation or deactivation of plant constituents involved with the ID increase was extremely fast since it reached near maximum after 2 min at 100°C.

Proteins have atoms on the side chains of amino acid moieties which can be extremely effective iron-binders, with many binding sites per molecule, e.g. 340 sites for a soy protein isolate (Galdi and Valencia, 1988). Most plant iron is bound to proteins (Price, 1968), especially as part of the chloroplasts redox system (Price, 1968). Proteins also undergo denaturation in a few seconds when denaturing agents are present. Subsequently, every change in protein structure due to denaturation may alter iron binding equilibria. In our boiling experiment, denaturation occured in all cases, as the 100°C was much higher than common protein denaturation temperatures (65–75°C). Protein denaturation can increase ID due to unfolding. Protein unfolding also renders proteins more susceptible to proteolytic enzymes (Rod-

Table 2—Soluble iron (SI) in simulated digestion of raw and cooked broccoli with and without addition of 17.5 ppm iron (as $FeCl_3$)[a]

	Raw broccoli		Cooked broccoli		Raw broccoli + $FeCl_3$		Cooked broccoli + $FeCl_3$	
	SI (ppm)	SI (%)	SI (ppm)	SI (%)	SI (ppm)	SI (%)	SI (ppm)	SI (%)
Vegetable+water	1.23 ± 0.03	30.7 ± 0.80	1.54 ± 0.03	38.50 ± 0.70	2.36 ± 0.06	10.99 ± 0.28	10.10 ± 0.1	46.98 ± 0.47
Pepsin Digest	0.17 ± 0.01	4.32 ± 0.26	2.36 ± 0.16	59.10 ± 4.01	0.49 ± 0.15	2.36 ± 0.70	18.30 ± 0.1	85.12 ± 0.46
Retentate	1.18 ± 0.13	39.47 ± 3.24	1.44 ± 0.05	56.03 ± 1.19	3.14 ± 0.08	18.60 ± 0.37	4.31 ± 0.01	33.33 ± 0.05
Dialyzate	0.22 ± 0.02	100*	0.69 ± 0.05	100*	0.34 ± 0.01	100*	4.41 ± 0.09	100*
Pancreatin-bile digest	1.40 ± 0.15	35.00 ± 3.63	2.13 ± 0.11	53.25 ± 2.38	3.48 ± 0.07	16.19 ± 0.33	8.72 ± 0.16	40.56 ± 0.74

[a] Iron concentration in broccoli: 7.99 ppm Fe; Each sample was tested in duplicate.
* No precipitate.

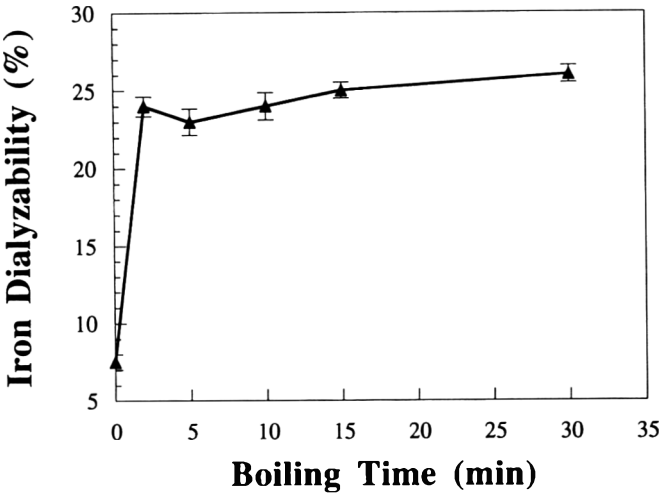


Fig.1—Effect of boiling time on the *in vitro* ID of iron from broccoli florettes.

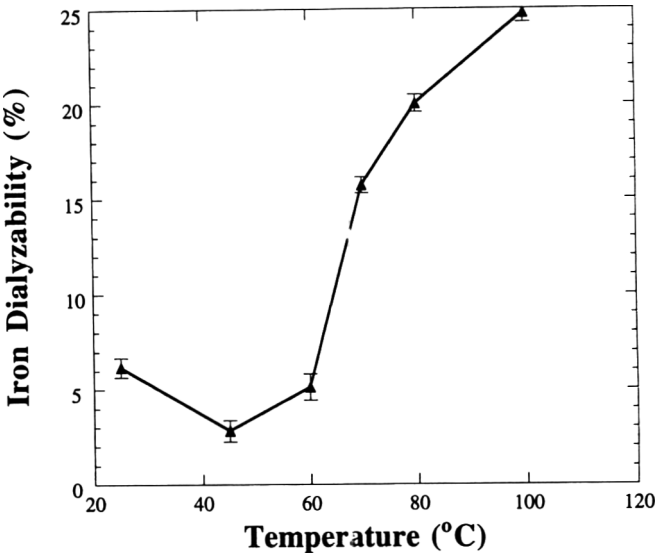


Fig. 2—Effect of heating (using hot plate) at various temperatures on the *in vitro* ID in broccoli florettes.

riguez et al., 1985) and causes faster release of soluble digestion products (amino acids, peptides) that can increase ID by complexing iron in low molecular weight complexes. Moreover, unfolding facilitates iron release and solubilization from protein complexes, predominantly by citric acid and secondarily by ascorbic acid and other chelators (Nadeau and Clydesdale, 1986).

Crucifers also contain considerable amounts of fiber (1–5 g/100 g), a component associated with iron binding and decreased iron absorption (Torre et al., 1991). Hemicellulose and lignin are major fiber components with high iron affinities, especially for pH>4 (Fernandez and Phillips, 1982a). With raw vegetables, cell wall material is intact and can bind iron on the inner and especially on the outer surface of plant cells. This may explain the lower ID of iron added to raw Crucifers. At the same time, organic acids are found in subcellular compartments (mainly in mitochondria as part of the Krebs cycle) which cannot solubilize iron from its complexes with fiber. However, heat treatment causes tissue softening, a combination of cell turgor pressure loss, air (extracellular and vascular) loss and denaturation (or partial degradation) of cell wall constituents (Haard, 1985). Tissue softening increases proteolysis, digestion and degradation of cellular and subcellular membranes facilitating release of enhancing factors (ascorbic acid, citric acid, sulfur-containing compounds) in the homogenate and increasing the ID of both intrinsic and extrinsic iron.

Effects of heating temperature

The effect of heating at different temperatures on ID showed a sharp and substantial increase of ID occurred between 60–80°C (Fig.2). This supported the hypothesis of the inhibitory role of proteins and fiber proposed above, as the temperature range of the increase coincided with the usual protein denaturation and tissue softening temperatures. Above 80°C, the rate of ID increase decreased with temperature. At 100°C, where denaturation was spontaneous and degradation of the cell wall material was substantial, ID reached the maximum. More studies at higher temperatures are needed to determine effects of HTST methods the use of which may result in high ID values without notable losses of other nutrients (e.g. vitamin C).

Microwave cooking

For microwave cooking effects on ID (Fig. 3), a trend similar to that with boiling was observed. The ID increase was very steep during the first 2 min (90 sec passed without boiling of the cooking water) and then a plateau was reached. This may indicate two heating mechanisms: direct heating of the protein and/or polysaccharides due to friction from the alternating magnetic field of microwave and indirect heating of protein by the water molecules (of vegetable and cooking medium) by conduction. This combination of heating mechanisms and high heating rate in the microwave oven was coupled with the high water content of the vegetables. This minimizes the time required for vegetable blanching, cooking, and processing thus retaining higher amounts of vitamins in processed foods (Salunkhe, 1974) without lessening the ID increase caused by heating. Therefore, use of microwave ovens for vegetable processing is generally considered desirable compared to conventional heat, water and steam methods.

Soluble iron (%SI) in various stages of *in vitro* digestion

Intrinsic iron in broccoli + water. Cooking of broccoli florettes resulted in 25% more SI in the initial homogenate (compared to that of raw broccoli). The increase may be attributed to liberation of enhancers that begin solubilizing iron from insoluble complexes.

Intrinsic iron in pepsin digest. SI differences became more pronounced after pepsin digestion. SI in cooked broccoli was

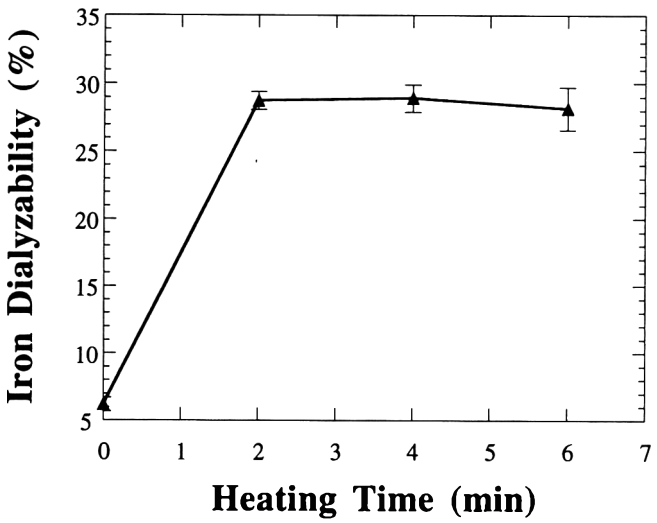


Fig. 3—Effect of microwave cooking on the *in vitro* ID of broccoli florettes.

14 times higher compared to uncooked. The difference was mainly due to a remarkable SI decrease from 31% in the initial homogenate (vegetable+water) to 4% in the pepsin digest of the raw vegetable. This was attributed to fiber and undigested protein that could bind iron before the enhancing factors started competition for the iron. On the contrary, SI increased in cooked vegetables (from 38% to 59%), due to generation of low molecular weight peptides (by proteolysis of denatured protein) and to the formation of soluble complexes with organic acids after being released from the mitochondria.

Intrinsic iron in PB digest, retentate, dialyzate. In raw broccoli, the initial insolubility resulted in a lower SI in the PB digest, as pH increased to 6.5 (intestinal environment) and slowed iron solubilization from protein and fiber. This solubilization occurred readily at acid pH (Fernandez, 1982a; Reinhold, 1982). Only 50% SI difference occurred between the PB digests; 3 times more iron was in the cooked sample dialyzate (consistent with the intrinsic ID experiment). This suggested that although there was appreciable SI in the retentate of raw broccoli, a small percentage of it had molecular weight low enough to dialyze. Insufficient protein digestion and/or iron solubilization from fiber may be the reason.

Extrinsic iron in broccoli+water. A large SI difference occurred among the initial homogenates (11% for the raw vegetable and 47% for cooked), an indication of immediate binding of added iron to raw vegetable. The binding occurred on fiber components on the outer cell wall side. On the other hand, protein denaturation and especially fiber degradation, coupled with release of enhancing factors in the cooked sample kept almost half the iron in soluble form(s).

Extrinsic iron in Pepsin digest. The difference became extreme after pepsin digestion where iron was essentially insoluble for raw broccoli (2.4%) and 85% soluble for cooked broccoli (38 times more SI). The same reasons indicated for the intrinsic iron experiment were valid here as well.

Extrinsic iron in PB digest, retentate, dialyzate. All the initial differences resulted in 12 times more iron in the dialyzate of the cooked vegetable and 2.5 times more SI in its pancreatin digest.

The similarity of trends in the SI results for intrinsic and extrinsic iron emphasized the similarity of absorption mechanisms between intrinsic and extrinsic iron. However, the inhibitory effects of fiber in the extrinsic iron experiment seemed more important than the effect of protein, since iron in that case had to initially interact with intact cell well material.

CONCLUSIONS

THE HIGH ID of cooked Crucifers or mixtures containing cooked Crucifers suggests that they contain iron of notable IB and could improve iron nutrition. The ability of cooked Crucifers to increase the solubility and ID of extrinsic NHI could be important for iron release from insoluble complexes with protein, fiber, polyphenols and other absorption inhibitors. This could result in increased IB from foods considered of low IB (soy isolate, cereal, legumes, etc.). If cooked Crucifers increased extrinsic iron bioavailability 3 fold (to around 30%), then the amount of dietary iron needed to satisfy the US RDA would be decreased. Cooking and general heating could greatly increase IB for intrinsic and extrinsic iron, even after short heat treatments. Studies on protein digestibility after heat treatments and determination of organic acid concentrations will further elucidate the mechanisms of ID increase. *In vivo* studies are needed for verification of potential of Crucifers in combating anemia.

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Frozen Carrots Texture and Pectic Components as Affected by Low-Temperature-Blanching and Quick Freezing

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ABSTRACT

Carrots preheated for 2 hr at 60°C and then cooked became firmer than raw or cooked carrots. After preheating, the amount of high methoxyl pectin decreased, and low methoxyl pectin increased. Firmness of carrots decreased through freezing then thawing, but preheated carrots retained firmer texture than those blanched in boiling water. Quick-freezing resulted in better texture than slow-freezing. Loss in texture was accompanied by release of pectin. Slow-freezing accelerated release of pectin as compared to quick-freezing. Preheated carrots were slower in release of pectin. The degree of esterification of pectin substances in raw carrots decreased during preheating, freezing and thawing. Cell damage in quick frozen carrots was slight. Optimum preheating occurred with 30 min at 60°C or 5 min at 70°C. Preheating and then quick freezing were effective in improving texture of frozen carrots.

Key Words: carrots, freezing, texture, preheating, pectin

INTRODUCTION

TEXTURE is an important quality attribute of frozen fruits and vegetables. A major result of freezing on fruits and vegetables is usually a loss of tissue firmness. Freezing causes disruption of the membranes of cells, and excessive softness (Rahman et al., 1971).

A correlation between pectic composition in cell walls and softening of cooked vegetables has been reported. Vegetables easily softened by thermal maceration contained more high methoxyl pectin than low methoxyl pectin (Fuchigami and Okamoto, 1984; Fuchigami, 1987a, b; Sajjaanantakul et al., 1989). Excessive softness could be overcome by low-temperature blanching. The de-esterification of pectin by pectin methylesterase (PME) during preheating prevented trans-elimination of pectin (Bartolome and Hoff, 1972b; Lee et al., 1979; Fuchigami, 1986; Quintero-Ramos et al., 1992). The firming effects of a low temperature, long-time treatment was useful for improvement in texture of frozen vegetables. When carrots were preheated for 30 min at 60°C, frozen at −30°C, thawed in a refrigerator and then cooked, softening of carrots was greatly diminished but cell damage was observed (Tamura, 1991). We reported that when carrots were frozen at −30°C, cell separation was great, thus the optimum rate of freezing for carrots was −5°C/min (Fuchigami et al., 1994). This suggested that when low-temp-blanched carrots are frozen at −5°C/min using a program freezer, histological change should be slight and softening should be minimized.

Our objective was to determine the effects of preheating carrots at 60°C and then quick freezing on texture improvement and cell damage of frozen carrots, and develop the most suitable temperature and time for preheating. Another objective was to investigate whether softening of frozen vegetables was related to changes in pectic composition of cell walls.

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MATERIALS & METHODES

Sample preparation

The cortex parenchyma of carrot roots (*Daucus carota L.* cv. Kuroda Gosun ninjin) grown in Okayama, Japan, were cut into 2g disks (11 mm thick and 15 mm diameter). The disks were divided into 17 lots for procedure -A and 60 lots for procecure -B, respectively. Three disks were used for each lot. Experiment was repeated two times.

Blanching, freezing and thawing

A—Effects of low-temperature-blanching and quick freezing on texture and pectic change. Disks were preheated for 2 hr at 60°C (low-temperature-blanching) or blanched for 3 min in boiling water (high-temperature-blanching). Raw, blanched or preheated disks were sealed into polyethylene bags and then frozen at −35°C for 2 days using a conventional freezer (Sanyo Medical Freezer, Sanyo Denki Co., Tokyo), or were also frozen with liquid nitrogen (freezing rate: −5°C/min, final temp. −50°C) using a program freezer (Cooling Master CM-Mark II, Taiyo Sanso Co., Tokyo). Disks were thawed for 30 min at 20°C or were also thawed for 3 min (raw and blanched disks) or for 6 min (preheated disks) in boiling water. For comparison, raw and preheated disks were cooled for 6 min in boiling water.

B—Effects of temperature and time of preheating on firmness. Disks were preheated at 60°C for 15, 30, 45 and 60 min, at 70°C for 5, 10, 15 and 30 min, at 80°C for 3, 5 and 10 min, at 85°C for 3, 4 and 5 min, at 90°C for 3 and 4 min, or at 100°C for 2, 3, 4 and 5 min. These preheated disks were frozen at −35°C for 2 days, and then thawed for 3 min in boiling water. For comparison, preheated disks were cooked for 3 min in boiling water.

Measurement of texture

Disks were cut horizontally into two pieces, and firmness and rupture strain of surfaces and inner parts of disks (from procedure A) were measured at 20°C using a creepmeter (RE-33005, Yamaden Co., Tokyo). Thickness of samples was measured using a sample-height counter, and then samples were compressed as the sample plate came up. The minimum gap between plunger (cylindrical shape, 2 mm diam) and sample plate was 0.01 mm. Maximum force (breaking load) was used as an index of firmness (N/m^2), and the distance of breaking point ÷ the thickness of sample × 100 was used as an index of rupture strain (%). The firmness of disks (treated by procedure B) was measured using a rheometer (NRM-2002J, Fudokogyo Co., Tokyo, interpenetrated type, cylindrical plunger; 2mm in diameter).

Extraction and determination of pectic substances

Pectic substances were extracted successively with four reagents: 0.01N HCl (pH 2.0, at 35°C, 24 hr×7), 0.1M sodium acetate buffer (pH 4.0, at 35°C, 24 hr×4), 2% sodium hexametaphosphate (pH 4.0, at 90°C, 3.5 hr×3), and 0.05N HCl (at 90°C, 4hr) solutions (Fuchigami and Okamoto, 1984; Fuchigami, 1987a, b). These fractions were designated as PA, PB, PC, and PD, respectively. DEAE-cellulose column chromatography of PA and PB was performed as reported previously (Fuchigami, 1987b). Galacturonic acid was determined by the carbazole method (Galambos, 1969) and natural sugars by the phenolsulfuric acid method (Dubois et al., 1956). Degree of esterification (DE) of pectic substances was measured by a gas chromatographic (GC) procedure (Bartolome and Hoff, 1972a). Monosaccharides were analyzed by a GC procedure (Kusakabe et al., 1977).

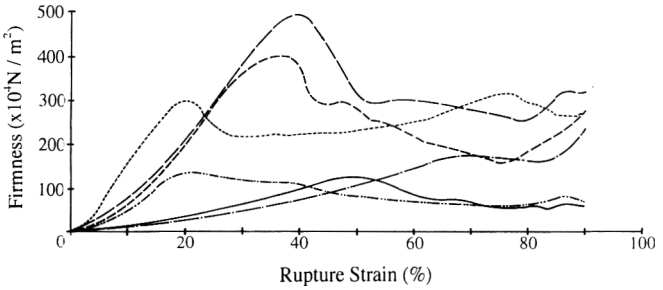


Fig. 1—Force-distance curves of raw, boiled, preheated, and frozen-thawed carrots. - - - - raw, — - - — boiled for 6 min, — — preheated for 2 hr at 60°C, - - - preheated then cooked for 6 min, — - - — preheated, frozen, then cooked for 6 min, —— blanched in boiling water, frozen, then cooked for 3 min. Carrots were frozen using a program freezer (−5°C/min).

Light microscope observation

From the center of thawed carrots, samples were cut in blocks of 1 mm^3. Tissue blocks were fixed, post-fixed, dehydrated, embedded, cut, and then stained for observation with a light microscope (Fuchigami et al., 1994).

RESULTS & DISCUSSION

Low-temperature-blanching and quick-freezing effects on texture

Typical force-distance curves of raw, boiled, preheated, preheated-then-cooked, and frozen-then-thawed carrots were compared using a creepmeter (Fig. 1). Raw disks broke when compressed to 20% of their thickness, but preheated carrots broke when compressed to 40% of initial thickness. Preheating increased firmness and rupture strain of carrots, while freezing-thawing decreased firmness and increased rupture strain.

The average of six measurements for firmness and rupture strain of nonfrozen and frozen-thawed carrots (raw, blanched or preheated) and significant differences (T-test) were compared (Tables 1 and 2). The disks boiled for 6 min softened ($p < 0.001$). However, those which were preheated for 2 hr at 60°C and then cooked for 6 min were firmer ($p < 0.001$) than either raw or boiled carrots.

Firmness of carrots decreased when frozen and thawed, except for raw carrots frozen in a program freezer (PF) and thawed. However, low-temperature-blanched carrots had firmer texture ($p < 0.001$) than high-temperature-blanched carrots after thawing. Raw and preheated disks frozen quickly (−5°C/min) using a PF were firmer than those frozen at −35°C using a conventional freezer (F), no difference occurred in firmness of blanched disks.

Rupture strains for raw, blanched or preheated carrots were about 20%, 30%, and 40%, respectively (Table 1). However, rupture strain increased after freezing and thawing ($p < 0.001$) (Table 2). Rupture strain (ca. 50~60%) of raw or blanched carrots frozen by PF was less than that (ca. 70~80%) frozen by F. However, that of preheated-frozen-thawed carrots was ≈80% (no differences between freezing rate).

Quick-freezing resulted in better texture (firmness and rupture strain) than slow-freezing, and also improved the texture of frozen carrots. Although low-temperature-blanching prevented softening of frozen carrots, it increased rupture strain. As a result, texture of frozen carrots differed from that of boiled carrots.

Table 1—Changes in firmness and rupture strain of carrots caused by blanching and cooking (n=6)

Sample	Firmness ($\times 10^4 N/m^2$)	Rupture strain (%)
1. raw	293 ± 61	20.6 ± 2.1
2. boiled for 3 min	252 ± 61	29.2 ± 2.6***
3. boiled for 6 min	140 ± 20***	26.2 ± 3.5**
4. preheated for 2 hr	558 ± 52***	41.4 ± 9.5***
5. preheated for 2 hr then cooked for 6 min	459 ± 61***	40.1 ± 5.6***

- $p > 0.05$, * $p<0.05$, ** $p<0.01$, *** $p<0.001$: significant differences between sample 1 and sample 2~5 (T-test).

Low-temperature-blanching and quick-freezing effects on pectic change

Changes in composition of pectic substances in carrots after blanching, preheating, freezing and thawing were followed (Fig. 2). The percentage of PA, PB, PC and PD in raw carrots was 45.5, 35.1, 17.2 and 2.2, respectively. During 6 min cooking, the amount of galacturonic acid in carrots decreased. After preheating at 60°C for 2 hr, the amount of PA decreased; conversely, PB increased.

Freezing and thawing accelerated pectin release, and slow-freezing (F) accelerated it also compared to quick-freezing (PF). The amount of galacturonic acid which remained in thawed disks was greatest to least; preheated > raw > blanched. Pectin composition did not change notably during freezing and thawing.

Changes in degree of esterification (DE) of pectic substances during blanching, preheating, freezing and thawing were followed (Table 3). PA in raw carrots was highly esterified (72.4%), while the DE of PB was lower (49.4%). Preheating reduced the DE of PA and PB to 58.7 and 36.4%, respectively, suggesting that the DE of pectic substances was decreased by PME during preheating (Bartolome and Hoff, 1972b). Freezing and thawing also decreased DE of PA and PB. Preheated carrots remained very firm after cooking for 6 min, and a great amount of pectic substances remained in them. This firming was caused by pectin de-esterification by PME, which inhibited pectic degradation by trans-elimination and accelerated binding with divalent cations such as Ca^{2+} (Fuchigami, 1986).

Table 2—Firmness and rupture strain of frozen then thawed carrots (n=6)

Sample	Freezing rate	Thawing (°C) (min)	Firmness ($\times 10^4 N/m^2$)	T-test	Rupture strain (%)	T-test
6. raw	fast[c]	20 30	331 ± 70 [1e]		56.4 ± 3.9***[1]	
7. raw	slow[d]	20 30	187 ± 29***[1]	***[6]	82.6 ± 3.2***[1]	***[6]
8. raw	fast	100 3	232 ± 68 [2]		58.9 ± 12.4***[2]	
9. raw	slow	100 3	153 ± 20***[2]	**[8]	81.6 ± 2.8***[2]	***[8]
10. blanched[a]	fast	20 30	131 ± 16***[2]		58.3 ± 6.4***[2]	
11. blanched	slow	20 30	119 ± 16***[2]	-[10]	73.0 ± 9.3***[2]	**[10]
12. blanched	fast	100 3	108 ± 23*[3]		50.0 ± 8.5***[3]	
13. blanched	slow	100 3	88 ± 18***[3]	-[12]	70.3 ± 12.5***[3]	**[12]
14. preheated[b]	fast	20 30	353 ± 56***[4]		81.3 ± 4.4***[4]	
15. preheated	slow	20 30	257 ± 16***[4]	***[14]	82.5 ± 5.1***[4]	-[14]
16. preheated	fast	100 6	252 ± 50***[5]		80.8 ± 7.6***[5]	
17. preheated	slow	100 6	200 ± 27***[5]	*[16]	81.0 ± 3.9***[5]	-[16]

[a] 3 min in boiling water.
[b] 2 hr at 60°C
[c] Program freezer (PF; freezing rate: −5°C/min, final temp. −50°C).
[d] Freezer (F) at −35°C.
[e] Significant differences by T-test (pair to sample number[1,2,3,4,5]), - $p > 0.05$, * $p<0.05$, ** $p<0.01$, *** $p<0.001$

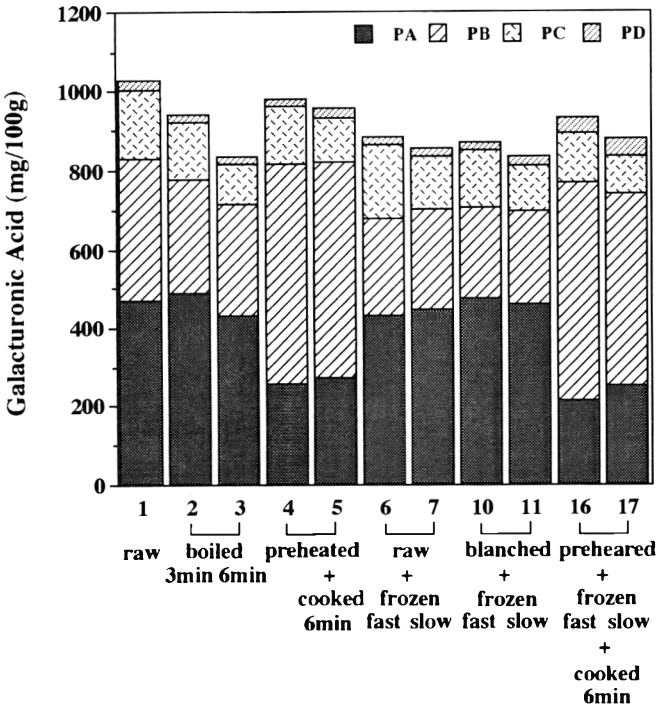


Fig. 2—Changes in pectic substances of carrots during blanching, preheating, freezing and thawing. 1~17: see Tables 1 and 2. PA: 0.01N HCl-soluble pectin, PB: acetate buffer-soluble pectin, PC: sodium hexametaphosphate-soluble pectin, PD: 0.05N HCl-soluble pectin.

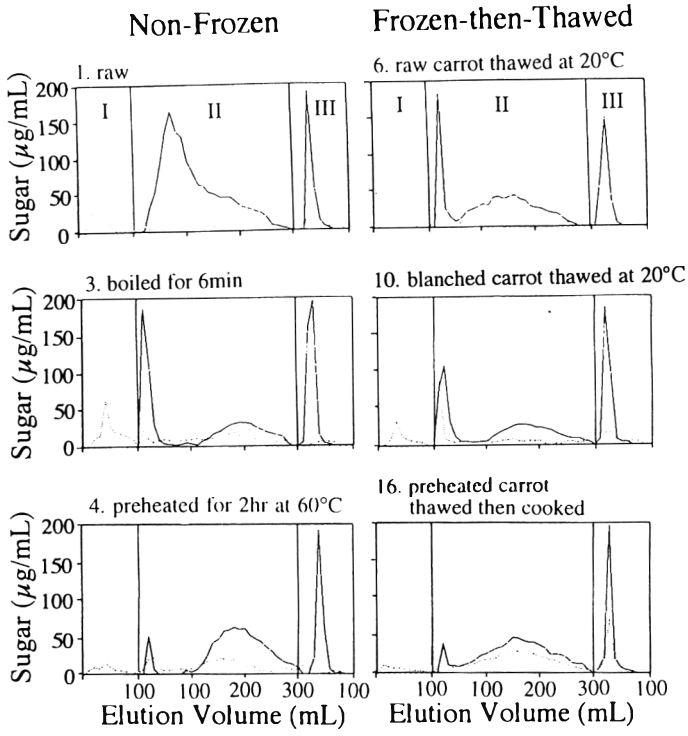


Fig. 3—Changes in DEAE-cellulose column chromatogram of HCl soluble-pectin (PA) in raw, blanched and preheated carrots during freezing and thawing. ——— galacturonic acid, - - - - - neutral sugar. I 0.02M acetate buffer, II gradient elution (0.1M → 1M acetate buffer), III 0.1N NaOH.

To investigate the properties of pectin in detail, PA and PB in raw, boiled and preheated carrots were fractionated into neutral and acidic fractions by DEAE-cellulose column chromatography. Changes in chromatograms during freezing-thawing were compared (Fig. 3 (PA) and 4 (PB)). Neutral sugar was eluted in a 0.02M acetate buffer (I). The next fraction (II) was eluted with a linear gradient of increasing concentration of acetate buffer (pH 6.0). Low methoxyl pectin was eluted later in this fraction. Finally, pectic substances with no methoxyl groups and a comparatively larger molecular weight were eluted with 0.1N NaOH (III) (Fuchigami, 1987b).

The PB fraction extracted from raw carrots eluted later in fraction II and in greater amounts in fraction III, when compared to PA fractionation. This proved that the DE of PB was lower than the DE of PA. When raw carrots were frozen by PF and then thawed at 20°C, the amount of PA eluted in fraction III increased. PB in frozen-then-thawed carrots eluted later in fraction II than PB in non-frozen carrots. This suggested that pectin DE decreased during freezing-then-thawing. Cell damage by freezing activated PME, then DE of pectin decreased during thawing at 20°C.

When carrots were boiled for 6 min, the comparatively high methoxyl pectin eluted at about 100 mL in fraction II decreased. These pectins were easily broken down by trans-elimination, and were released into boiling water. But there was little change in chromatograms of PA and PB during freezing-thawing.

When carrots were preheated for 2 hr at 60°C, the amounts of PA eluted in fraction III and PB eluted later in fraction II increased in comparison to raw carrots. This suggested that preheating at 60°C decreased the DE of PA and PB. Freezing-then-thawing did not appreciably change the chromatograms of PA and PB. Preheating at 60°C not only decreased PA and increased PB, but also decreased the DE of PA and PB.

The composition of neutral sugars of PA and PB in raw carrots was compared (Table 4). Arabinose in PA was 71.8%, and galactose in PB was 58% of the neutral sugars in fraction I. About 70% of neutral sugars in fraction II of PA and PB, was galactose.

Effects of temperature and time of preheating on firmness

Effects of temperature and time of preheating on firmness of frozen carrots was determined (Fig. 5). Firmness of carrots preheated at various temperature was, greatest to least: 70°C > 60°C > 80°C > 85°C > 90°C > 100°C. Carrots preheated above 85°C softened quickly as temperature rose.

Carrots preheated at 60°C or 70°C and then cooked for 3 min were much firmer than those cooked without preheating (control). Carrots preheated for 5 min at 70°C or for 30 min at 60°C before cooking were firmest and maintained firmness after extension of preheating time. When carrots were preheated at 80°C for 10 min, at 85°C for 5 min or at 90°C for 3 min, and then cooked, they were firmer than the control. The PME might be activated at these temperatures for a very short time.

When carrots preheated under the same conditions were frozen at −35°C and then cooked for 3 min, firmness decreased in

Table 3—Change in degree of esterification of pectic substances during blanching, preheating, freezing and thawing (%)

	Nonfrozen					Frozen-then-thawed (20°C)				Frozen-thawed-cooked	
	Raw	Boiled		Preheated		Raw		Blanched		Preheated	
Sample no.[a]	1	3 min 2	6 min 3	4	Cooked 5	Fast 6	Slow 7	Fast 10	Slow 11	Fast 16	Slow 17
PA	72.4	66.5	58.0	58.7	57.7	61.8	58.6	60.0	62.6	54.4	50.4
PB	49.4	45.1	38.3	36.4	38.5	45.3	42.7	49.3	42.2	35.9	35.5

[a] Sample number: see Tables 1 and 2. PA, PB: see Fig. 2.

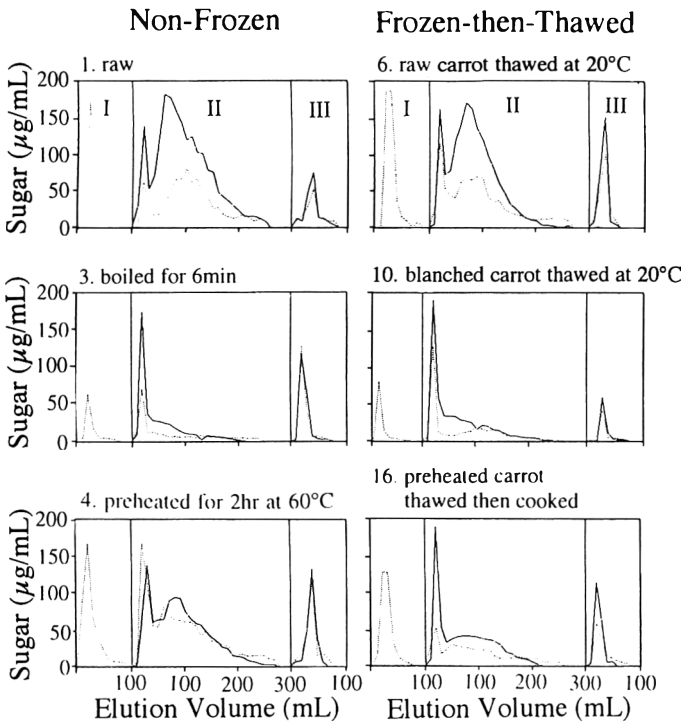


Fig. 4—Changes in DEAE-cellulose column chromatogram of acetate buffer-soluble pectin (PB) in raw, blanched and preheated carrots during freezing and thawing. Symbols: see Fig. 3.

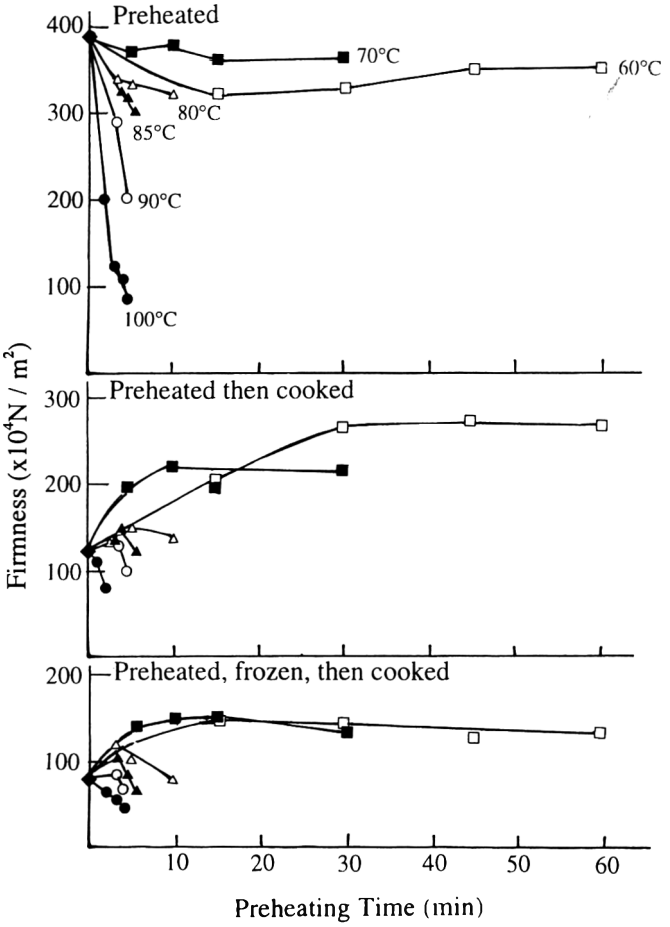


Fig. 5—Effects of temperature and time of preheating on the firmness of frozen carrots.

comparison to preheated-then-cooked carrots. The firmness of preheated-frozen-cooked carrots showed the same tendency as the firmness of preheated-cooked carrots. Those preheated for 30 min at 60°C and for 5 min at 70°C maintained firmness after freezing-thawing-cooking. The optimum preheating time at 60°C was 30 min, because longer soaking times in 60°C water caused loss of taste (such as sweetness).

Low-temperature-blanching and quick-freezing effects on histological changes

Histological changes in light microscopic observations of preheated carrots after freezing-then-thawing were compared (Fig. 6). When carrots were boiled for 6 min, cell separations of the middle lamella were observed (3), but they were not observed in carrots preheated for 2 hr at 60°C (4). Carrots preheated-then-cooked for 6 min macerated slightly (5).

When preheated carrots were frozen at −35°C and then thawed at 20°C (15) or 100°C (17), cell damage was apparent. However, when they were frozen by PF (−5°C/min) and thawed at 20°C (14) or 100°C (16), cell damage was minimal.

When carrots preheated for 30 min at 60°C (a) or for 5 min at 70°C (c) were frozen by PF and then cooked for 3 min, cell damage was comparatively slight. To prevent the activity of PME and oxidase, carrots cooked for 3 min after preheating (Steinbuch, 1976) were then frozen by PF and thawed at 20°C. Cell damage in those samples was also slight, but cell separation in carrots preheated at 70°C (d) was greater than in those preheated at 60°C (e).

CONCLUSIONS

CARROTS PREHEATED AT 60°C and frozen quickly by a program freezer (−5°C/min) maintained greater firmness and had less cell damage than high temperature blanched carrots frozen slowly by a conventional freezer (−35°C). Degree of esterification of pectin decreased during preheating, freezing and thawing. Thus, preheating prevented release of pectin. Preheating for 30 min at 60°C and then quick freezing were effective in improving the texture of frozen carrots.

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Table 4—Monosaccharide composition of polysaccharide fractions of raw carrots separated by DEAE-cellulose column chromatography

Pectin	Fraction by DEAE[a]	Composition of monosaccharides (%)					
		Rhamnose	Arabinose	Xylose	Mannose	Galactose	Glucose
PA	I	0.7	71.8	1.6	2.2	23.1	0.6
	II	2.2	26.4	0.9	2.2	68.3	0
	III	4.0	35.0	7.7	8.3	22.0	23.0
PB	I	1.4	16.2	0	15.4	58.0	9.0
	II	4.0	21.8	1.4	1.1	71.7	0
	III	trace	40.0	trace	11.5	18.9	29.6

[a] See Fig. 3.

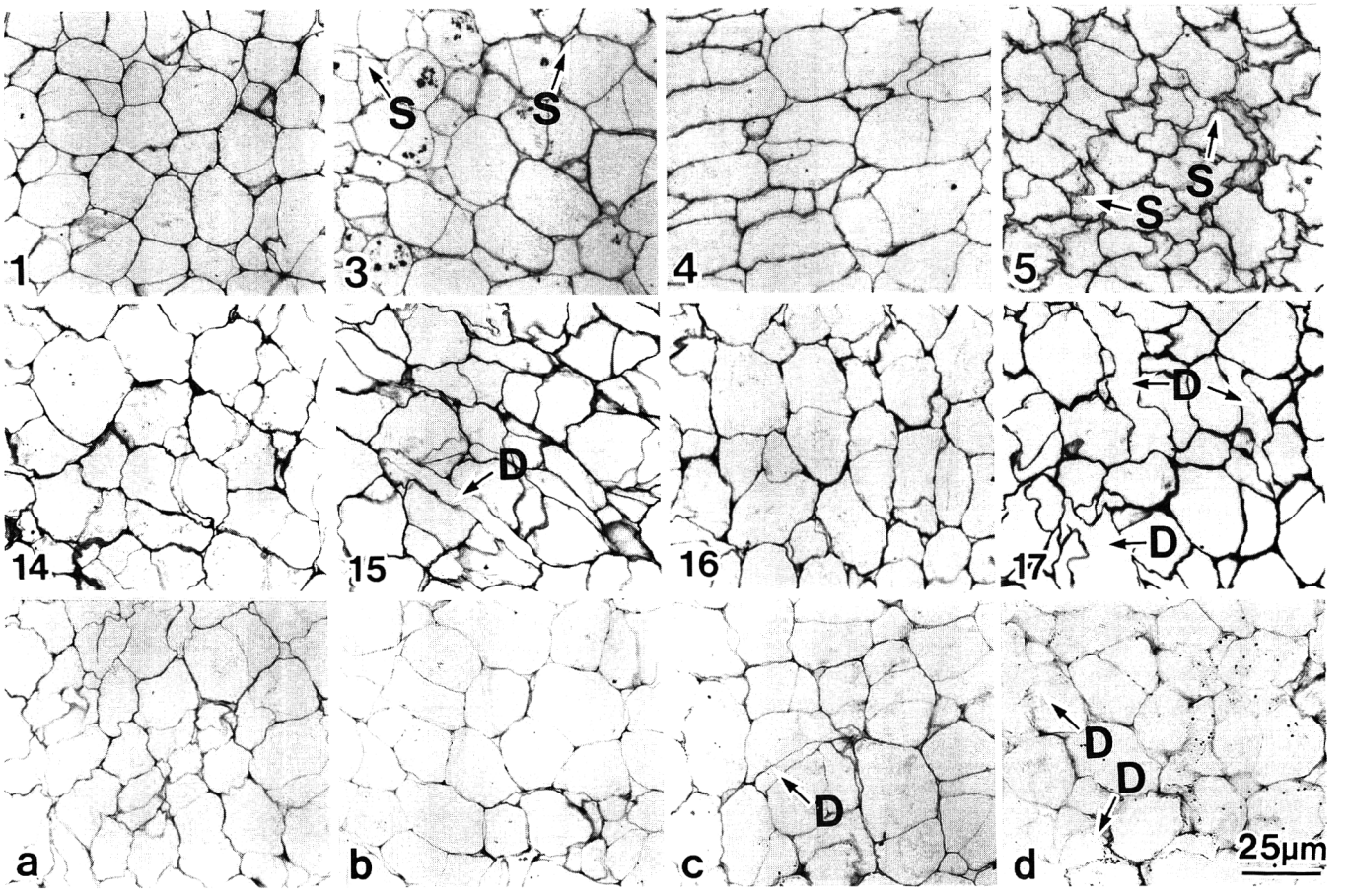


Fig. 6—Light micrographs showing the parenchyma of raw, boiled, preheated, preheated-then-cooked, and preheated-frozen-then-thawed carrots. Number: see Tables 1 and 2. (a) preheated for 30 min at 60°C, frozen by PF, and then cooked for 3 min. (b) preheated for 30 min at 60°C, then cooked for 3 min, frozen by PF, and thawed at 20°C. (c) preheated for 5 min at 70°C, frozen by PF, and then cooked for 3 min. (d) preheated for 5 min at 70°C, then cooked for 3 min, frozen by PF, and thawed at 20°C. PF: see Table 2. S: cell separation, D: cell damage.

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Programmed Freezing Affects Texture, Pectic Composition and Electron Microscopic Structures of Carrots

MICHIKO FUCHIGAMI, NORIKO HYAKUMOTO, and KOICHI MIYAZAKI

ABSTRACT

Raw and blanched carrots (3 min, boiling water) were frozen at −2°C, −3°C, −4°C or −5°C/min (final −20°C or −50°C) then thawed at 20°C or 100°C. Firmness of thawed raw carrots was: −5°C > −4°C > −3°C > −2°C/min. Effect of freezing rate on blanched carrots was less than that on raw carrots, but firmness of thawed carrots was not affected by final temperature of freezing. When raw carrots were thawed at 20°C, high methoxyl pectin decreased. Pectin decrease in blanched carrots caused by freezing was greater than that in frozen raw carrots. Effects of slow-freezing, programmed-freezing (slow + quick + slow) and quick-freezing showed quick freezing (−5°C/min) best for texture. As freezing rate decreased, drip increased. A wide difference among experimental samples in fine structure was revealed by cryo-scanning electron microscopy.

Key Words: carrots, programmed freezing, pectic composition, electron microscopy.

INTRODUCTION

TEXTURAL QUALITY OF FRUITS AND VEGETABLES LESSEN UPON freezing and thawing, resulting in excessive softening (Reeve, 1970; Rahman et al., 1971; Goodenough and Atkin, 1981; Zeuthen et al., 1984; Bald, 1991). It is more difficult to produce frozen vegetables with good textural quality than to produce quality frozen fish and meat.

The rate of freezing is recognized as critical in tissue damage (Rahman et al., 1971). Carrots immersed in liquid nitrogen (LN_2) cracked, but those frozen at −5°C/min or −2°C/min using a programmed freezer (final temp, −30°C) had better texture than those frozen in a conventional freezer at −80°C, −30°C or −20°C (Fuchigami et al., 1994). Our first objective was to determine the optimum rate of freezing (−5°C, −4°C, −3°C and −2°C/min) and the final freezing temperature (−20°C or −50°C) for improvement in texture of frozen carrots, and to determine pectic changes during freezing-thawing.

Pectic composition (methyl ester content) affected softening of vegetables during cooking (Fuchigami, 1987a; Sajjaanantakul et al., 1989). Carrots preheated at 60°C and then cooked became firmer than raw or cooked carrots. The release of pectin decreased with de-esterification by pectin methylesterase (PME) (Quintero-Ramos et al., 1992; Fuchigami et al., 1995). Therefore, low-temperature blanching effectively improved texture of frozen carrots.

For improvement in texture of frozen foods such as meat, fish, sushi, or rice cake, the capsule-packed-freezing method (CPF method) was applied (Mitsuda and Ueno, 1983; Mitsuda et al., 1982). In this method, materials were cooled for 5 ~ 10 min at −80 ~ −100°C using LN_2, and ice-capsules (which resist expansion by freezing) were formed around the food surface. Then the food was cooled to 0°C for 5 ~ 30 min at −25 ~ −35°C and frozen at −80°C ~ −100°C. After the central temperature of the food reached −6 ± 1°C, they were frozen slowly for 40 ~ 90 min at −25 ~ −35°C, and then stored at −18°C. With this method, frozen foods had good textural qualities. Our second objective was to determine whether such programmed freezing methods could be applied to frozen vegetables.

In previous papers (Fuchigami et al., 1995), histological changes in frozen carrots were reported with a light microscope (LM) and a transmission electron microscope (TEM). Cell damage was great when freezing rate was slow. However, details of primary cell walls and middle lamella were not clear, because cellulose micrifibrils were not stained by uranium and lead. To observe more detail, a scanning electron microscope (SEM) was used. Plant tissues (high moisture containing tissues) generally have been difficult to prepare for SEM. The cryofractured-critical point drying technique for raw and cooked carrot phloem and xylem was less damaging (Davis et al., 1976a,b; Davis and Gordon, 1980). However, specimen preparation was complicated, and it was difficult to observe at high magnification. These problems were solved using a high resolution SEM with cryo-system which showed molecular detail. Our third objective was to compare the fine structures of raw, cooked and quick- or slow-frozen-thawed carrots using that technique.

Table 1—Rate of freezing (°C/min)

Temp (°C)	Slow	Programmed			Quick	
	SF	PF1	PF2	PF3	−2°C	−5°C
10 → 0	−0.5	−0.5	−0.5	−0.5	−2	−5
0 → −2.5	−0.25[a]	−2	−2	−5	−2	−5
−2.5 → −5	−0.25	−2	−2	−5	−2	−5
−5 → −10	−0.5	−0.5	−2	−5	−2	−5
−10 → −20	−0.5	−0.5	−0.5	−0.5	−2	−5

[a] Temperature was maintained for 90 min at −2.5°C.

MATERIALS & METHODS

Sample preparation

Cortex parenchyma of carrot roots (*Daucus carota* L. cv. Kuroda Gosun Ninjin) grown in Okayama were cut into 2g disks (15 mm diam and 11 mm thick). Both raw disks and blanched disks (boiled 3 min in distilled water) were used. Three disks were used for one lot, and experiments were repeated two times.

Methods of freezing and thawing

Samples were frozen at −5°C, −4°C, −3°C, or −2°C/min (final temp, −50°C or −20°C) using a programmed freezer (CM-Mark II, Taiyo Sanso Co.) with liquid nitrogen (LN_2) (Fuchigami et al, 1995). Samples were also frozen in six different ways (Table 1 and Fig. 6) using a program freezer: Slow-Freezing (SF), Programmed-Freezing (slow + quick + slow freezing: PF1, PF2 and PF3) and Quick-Freezing (−5°C/min and −2°C/min). Frozen carrots were thawed 30 min at room temperature (20°C), or for 3 min in boiling distilled water (800 mL).

Measurement of texture

Thawed disks were cut in a horizontal direction into two pieces; firmness of surfaces and inner parts of disks were measured using a rheometer (NMR-2002J, Fudo Kogyo Co. Tokyo). A plunger of cylindrical shape (2 mm in diam) was used. Crosshead speed was 30 cm/min.

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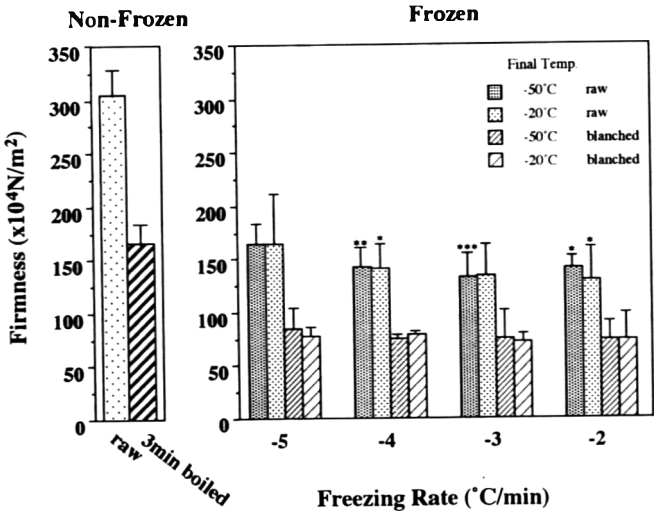


Fig. 1—Effects of the rate and the final temperature of freezing on firmness of frozen carrots thawed at 20°C. Means ± standard deviations (n = 6). * $p < 0.05$, ** $P < 0.01$, *** $p < 0.001$. There were significant differences between −5°C/min and −4, −3, −2°C/min (T-test).

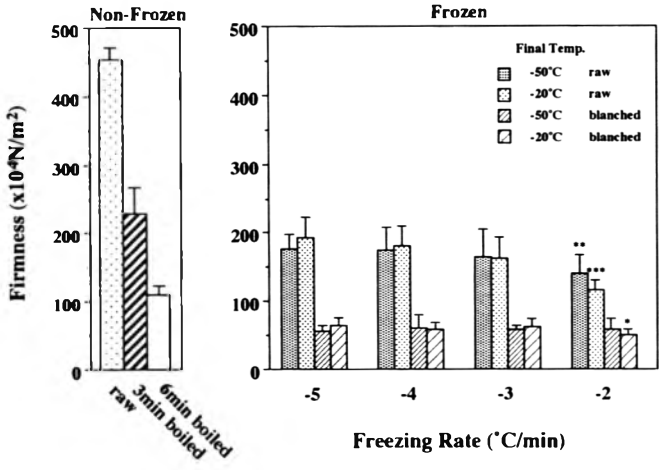


Fig. 2—Effects of rate and final temperature of freezing on firmness of frozen carrots thawed in boiling water. *, **, ***. See Fig. 1.

Extraction and determination of pectic substances

Alcohol-insoluble solids (AIS) were prepared from raw, boiled, and frozen-then-thawed carrots. Pectic substances were extracted from AIS through successive extractions with five reagents: distilled water (at 20°C, 12 hr × 2), 0.01N HCl (at 35°C, 24 hr × 7), 0.1M sodium acetate buffer (at 35°C, 24 hr × 4), 2% sodium hexametaphosphate (at 90°C, 3.5 hr × 3), and 0.05N HCl (at 90°C, 4hr) solutions. These fractions were designated as WSP, PA, PB, PC, and PD, respectively (Fuchigami and Okamoto, 1984, Fuchigami, 1987a). The galacturonic acid in the 5 fractions was determined by the carbazole method (Galambos, 1969).

Cryo-SEM observation

Raw, cooked for 6 min or 30 min, and raw-frozen (PF −5°C/min or −30°C freezer)-then-thawed (at 20°C) carrots were cut into 6mm × 1mm × 1mm, and dehydrated with 20%, 40% and 50% ethanol. Specimens were contained in metal holders and quickly frozen by immersing in LN_2. Frozen specimens were transferred to the cold stage of a cryo-system for scanning electron microscopy (S-4200, Hitachi Co., Tokyo), and then cut with a knife (−150°C). After etching at −85°C, the surface was observed at −120°C under low acceleration voltage (1kV).

RESULTS & DISCUSSION

Effects of rate and final temperature of freezing on texture

Disks were frozen linearly along a freezing line entered by computer using a program freezer, and the freezing point was passed through rapidly (Fuchigami et al., 1995). Effects of rate and final temperature of freezing on firmness of frozen carrots thawed at 20°C or thawed in boiling water were compared (Fig. 1 and 2). Firmness of carrots decreased ($p < 0.001$) through freezing-then-thawing. After thawing at 20°C or 100°C, firmness of raw carrots was greatest to least: −5°C/min > −4°C/min > −3°C/min > −2°C/min. A significant difference occurred between −5°C/min and −2°C/min. However, no differences occurred in firmness of blanched-frozen-thawed carrots due to freezing rate. The effect of freezing rate on firmness of raw carrots was greater than that on firmness of blanched carrots. The final temperature of freezing did not affect firmness of thawed carrots.

The amount of drip after thawing at 20°C and 100°C was about 4% and 17 ~ 26%, respectively (Fig. 3). When frozen carrots were thawed in boiling water, the amount of drip increased when freezing rate was slow. However, a difference in drip during freezing rate was not observed after thawing at 20°C.

Effects of rate and final temperature of freezing on pectic composition

Raw or blanched carrots were frozen at −5°C/min or −2°C/min (final temperature: −20°C or −50°C). Pectic changes during freezing and then thawing at 20°C were compared (Fig. 4 and 5). When carrots were frozen and then thawed, amount of pectic substances decreased. This decrease in blanched carrots was greater than in raw carrots.

Pectins of carrots were fractionated with five reagents. The uronic acid distribution of WSP, PA, PB, PC and PD in raw carrots was about 1%, 39%, 36%, 20% and 4%, respectively. Water-soluble pectin (WSP) was extremely low. While dipping tissues in 0.01N HCl (pH 2.0) at 35°C, 38% pectin (PA) was extracted due to removal of polyvalent cations (Fuchigami and Okamoto, 1984). These results suggested that pectic substances in cell walls seemed to be insoluble because of polyvalent cations such as Ca^{2+} (Fuchigami, 1987b). Low-methoxyl pectin was usually precipitated at pH 2, therefore, PA was high-methoxyl pectin (degree of esterification: DE = 72%). Low-methoxyl pectin (PB) was successively extracted with acetate buffer (pH 4) at 35°C (DE = 49%) (Fuchigami et al., 1994). PC and PD extracted at 90°C were also low-methoxyl pectin.

When carrots were boiled for 3 min or 6 min in distilled water (pH 6.5), pectins were degraded by transelimination and released into the cooking solution. The amount of WSP remaining in the carrots increased. The WSP was easily extracted because of degradation of pectin. After freezing-thawing, the amount of WSP in samples which were blanched or thawed at 100°C was greater than that in raw carrots thawed at 20°C.

Changes in pectic composition during freezing-thawing were comparatively slight. The PA in raw carrots frozen-thawed at 20°C decreased slightly. These results suggest that DE of pectin decreased during freezing and thawing. When cells are damaged through freezing, PME might be activated during thawing at 20°C (Bartolome and Hoff, 1972, Fuchigami et al., 1995).

No difference occurred in the total pectins which remained in carrots frozen at −5°C/min and those frozen at −2°C/min. As rate of freezing was slow, the amount of pectin in drip increased. This suggested that cell damage during freezing accelerated release of pectin into the cooking solution. The final temperature of freezing had little effect on pectic change.

Effect of programmed freezing on firmness

Changes in temperature of carrots during slow-freezing, various programmed-freezing regimes and quick-freezing were

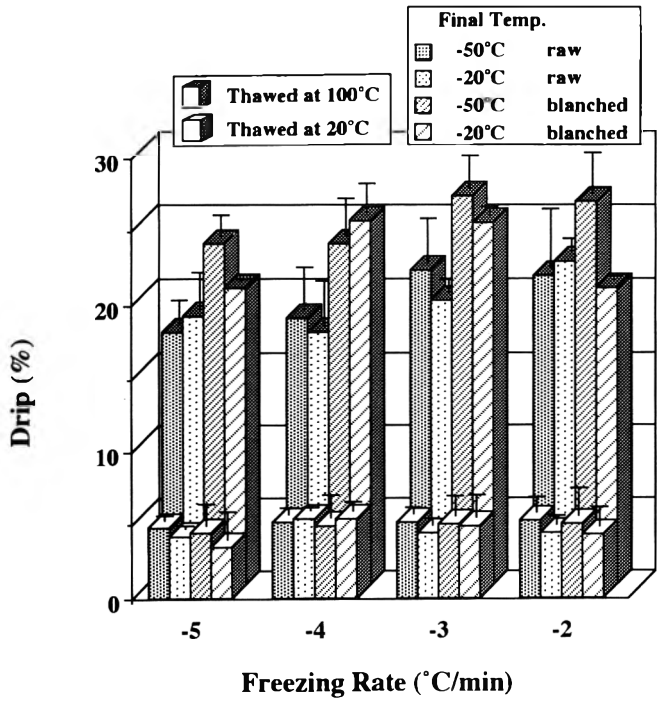


Fig. 3—Effects of rate and final temperature of freezing on amount of drip.

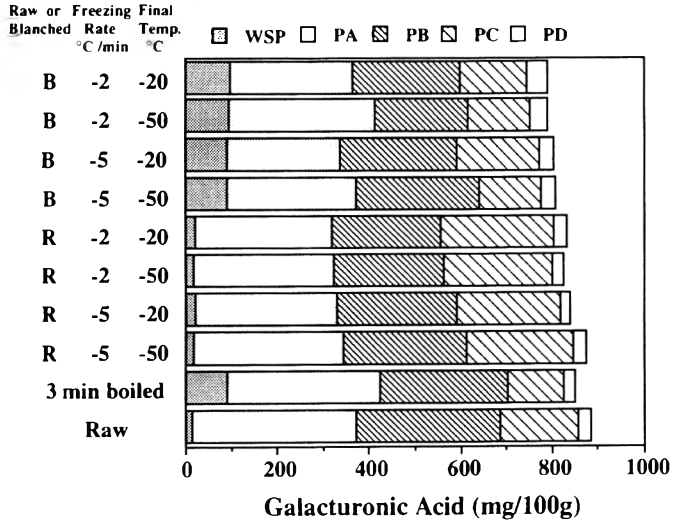


Fig. 4—Effects of rate and final temperature of freezing on pectic composition of frozen carrots thawed at 20°C. WSP: water-soluble pectin, PA: 0.01N HCl-soluble pectin, PB: acetate buffer-soluble pectin, PC: sodium hexametaphosphate-soluble pectin, PD: 0.05N HCl-soluble pectin.

compared (Fig. 6). Firmness of frozen carrots and amount of drip when thawed at 20°C were also compared (Fig. 7). Firmness of frozen-thawed raw carrots was least to greatest: SF < PF1 < −2°C/min < PF2 < PF3 < −5°C/min. Differences occurred between PF1 and PF2, PF3 ($p < 0.01$), −5°C/min ($p < 0.001$); between −5°C/min and −2°C/min ($p < 0.001$). As freezing rate from 0°C to −10°C was quick, firmness of thawed carrots was maintained. The amount of drip increased as freezing rate decreased.

Effect of freezing rate on firmness of blanched carrots was the same as that of raw carrots. However, the degree of effect on blanched carrots was smaller than that on raw carrots. Consequently, quick freezing (−5°C/min) was best for texture of frozen carrots.

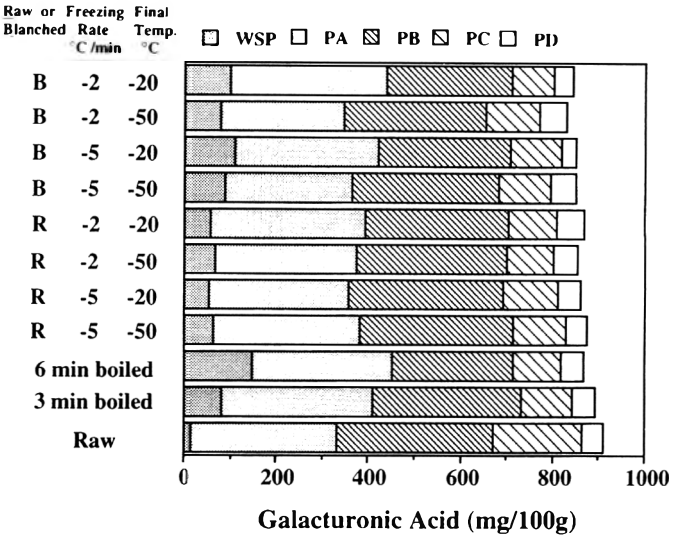


Fig. 5—Effects of rate and final temperature of freezing on pectic composition of frozen carrots thawed in boiling water. WSP, PA, PB, PC, and PD: See Fig. 4.

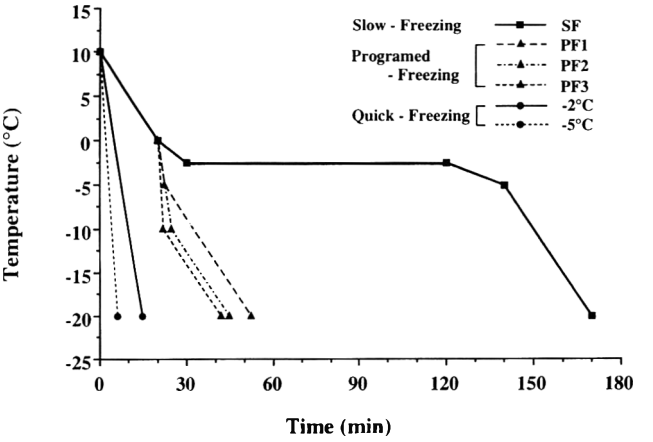


Fig. 6—Changes in temperature of carrots during slow-freezing, programmed-freezing and quick-freezing using a program freezer.

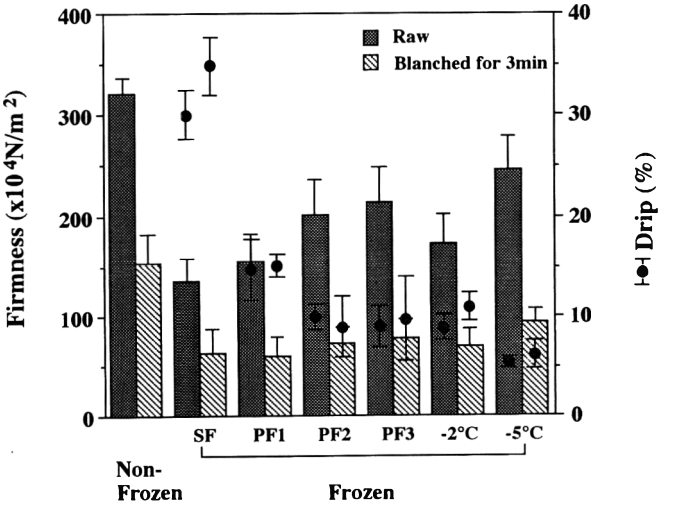


Fig. 7—Effects of programmed-freezing on firmness of frozen carrots thawed at 20°C. SF, PF1, PF2, PF3, −2°C, and −5°C: See Fig. 6.

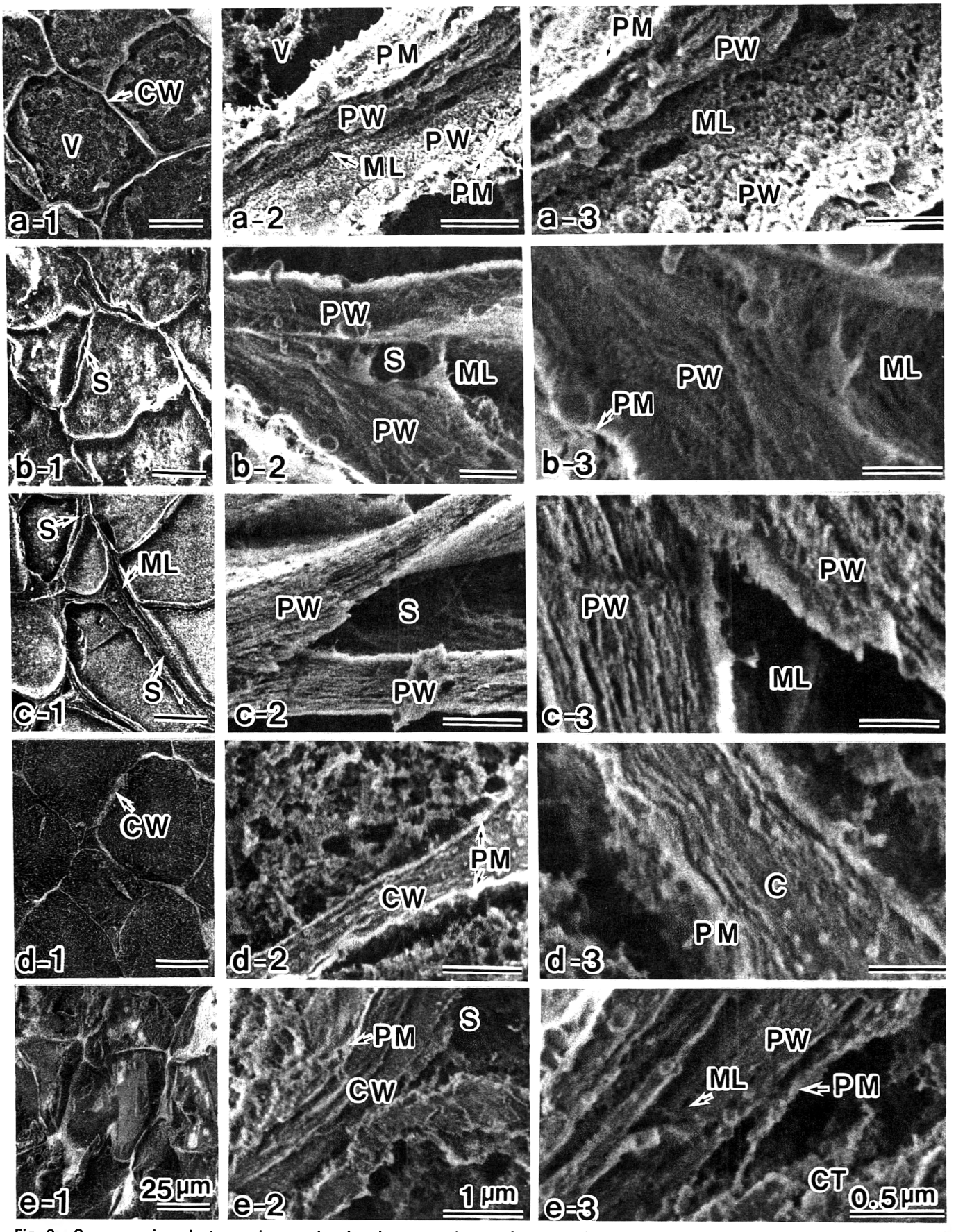


Fig. 8.—Cryo-scanning electron micrographs showing parenchyma of raw, cooked and frozen-thawed carrots. (a) raw carrot; (b) carrot cooked for 6 min; (c) carrot cooked for 30 min; (d) raw carrot frozen at −5°C/min using a program freezer, then thawed at 20°C; (e) raw carrot frozen at −30°C using a conventional freezer, then thawed at 20°C; [1] ×400, bar 25 μm; [2] ×10,000 ~ 15,000, bar 1 μm; [3] ×30,000 ~ 35,000, 0.5 μm. CT: cytoplasm; CW: cell wall; ML: middle lamella; PM: plasma membrane; PW: primary wall; S: separated region of cell wall; V: vacuole.

Cryo-SEM observations of raw, cooked and frozen-thawed carrots

Histological changes in Cryo-SEM observations (×400, ×10,000 ~ 15,000, ×30,000 ~ 35,000) of raw, cooked and frozen-thawed carrots were observed (Fig. 8). Cell walls of raw carrots were observed in white (a-1). Plasma membranes were also observed in white and cemented cell walls (a-2). The middle lamella (pectin rich) appeared dark at the center of primary walls (a-2). The high magnification micrographs of raw carrots showed reticular structures with granular components in the middle lamella and primary cell walls (a-2, a-3). The granular components might be pectic substances according to observation by quick-freeze, deep-etch electron microscopy (Tamura and Senda, 1992).

After cooking for 6 min, cell separation in the middle lamella due to solubilizing of pectic substances was observed (b-1 ~ 3). The high magnification micrographs showed fibrous components (long cellulose microfibrils) in primary cell walls. However, reticular structures and granular components which were found in raw cell walls were not observed. Large differences were observed between raw and cooked cell walls. After 30 min cooking, the degree of cell separation in the middle lamella and the spaces among microfibrils increased (C1 ~ 3). Raw primary cell walls consist of microfibrillar phase and matrix. After cooking, pectic substances in the matrix were removed; consequently, the microfibrillar phase became loose.

When raw carrots were frozen at −5°C/min, then thawed at 20°C, cell damage was not observed and plasma membranes cemented cell walls (d-1 ~ 3). However, after freezing at −30°C using a conventional freezer, cell damage was observed (e-1 ~ 3), and the plasma membrane separated from the cell wall. These results were similar to those reported with LM and TEM (Fuchigami et al., 1994b). Cryo-SEM results reconfirmed that the optimum rate of freezing for carrots was −5°C/min.

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Sensory, Microbiological and Chemical Quality of Mini-Peeled Carrots as Affected by Edible Coating Treatment

L.R. HOWARD and T. DEWI

ABSTRACT

The effect of three application rates of an edible cellulose-based coating on sensory, microbiological and chemical quality of mini-peeled carrots was studied during storage at 2°C. Carrots treated with edible coating had reduced levels of white surface discoloration, and higher sensory scores for orange color intensity, fresh carrot aroma, fresh carrot flavor, and overall acceptability than non-coated carrots. Edible coating treatment did not affect levels of total aerobic microorganisms, yeast, mold or lactobacilli. Oxygen, carbon dioxide and ethanol levels in packages, and sugar and soluble phenolic content of mini-peeled carrots were not affected by coating treatment. An edible coating application rate of 0.23–0.49 L/min protected against surface discoloration and retained quality during storage at 2°C.

Key Words: carrots, minimal processed, edible coating, sensory, microbial quality

INTRODUCTION

MINIMALLY PROCESSED VEGETABLES provide convenience, fresh characteristics and human health benefits. Minimal processing may include any unit operations (washing, sorting, peeling, coring, slicing etc.) used prior to blanching in a conventional processing line. Some fresh carrots are minimally processed by abrasive peelers and washed to remove cellular fluids to produce carrot sticks, baby peeled carrots and grated carrots. The shelf life of packaged minimally processed carrots is sometimes limited by a white discoloration that develops on abraded surfaces during storage, which consumers may associate with aging. White discoloration may be due to formation of lignin as a wound barrier (Bolin and Huxsoll, 1991; Howard and Griffin, 1993) and/or dehydration of abraded surfaces (Tatsumi et al., 1991). Surface discoloration may be controlled by dipping in heated acid or alkali solutions (Bolin and Huxsoll, 1991; Bolin, 1992), exposing abraded surfaces to steam (Howard et al., 1994a), or application of polymer treatments (Bruemmer, 1987; Springler et al., 1989; Krochta et al., 1993). Edible coatings placed on abraded surfaces of minimally processed fruits and vegetables may retard product dehydration, create an internal atmosphere to retard senescence and/or act as a barrier against spoilage and pathogenic microorganisms (Kester and Fennema, 1986; Krochta et al., 1988). An ideal treatment for mini-peeled carrots would control surface discoloration without affecting color, flavor, texture or microbial quality of the product.

Our objective was to determine the effects of an edible cellulose-based coating on sensory, chemical and microbiological quality of mini-peeled carrots.

MATERIALS & METHODS

Product preparation and processing

The experiment was conducted at McManus Produce Company, Weslaco, TX 78596. Nature Seal™ 1000, an edible cellulose based polymer (Nisperos-Carriedo and Baldwin, 1993) with a molecular weight range of 400,000–800,000 (EcoScience Corp., Worcester, MA) was applied to mini-peeled carrots using a Solo Model 425X sprayer at ≈1mg/cm². Different nozzle sizes were used to provide three application rates. Teejet nozzles 8001 and 8003 resulted in application rates of 0.23 and 0.49 L/min while Lurmark nozzle 04F110 resulted in an application rate of 0.87 L/min. Edible coating was applied for 2–3 sec to mini-peeled carrots (21.8 kg/min) as they dropped onto a vibratory table prior to bagging. Mini-peeled carrots (454g) were placed into a 1.5 mil polyethylene 370 film (James River Corp., Cincinnati, OH), and the packages were heat sealed. The packaging film had a surface area of 723 cm², and an oxygen transmission rate of 5317 cc/m²/24hr. The first cases of carrots treated with each application rate were discarded to ensure uniform application. The next two cases (24 bags/case) were collected for the experiment. Two cases of control samples, (no coating) were collected from the processing line prior to coating. Samples were transported to Texas A&M University in coolers the same day they were processed and randomly stored on racks at 1.7°C. Samples were removed 0, 1, 2, 3, 4, and 5 wks after treatment. Three replicates were used for analysis of headspace gases and microbiological determinations with each bag of carrots serving as a replication. Five mini-peeled carrots from each bag (three bags/treatment) were used for color determination. Three bags of mini-peeled carrots were used for each sensory evaluation. For sugar and total soluble phenolic determination ≈20 carrots from each bag (three bags/treatment) were shredded and mixed thoroughly. Three subsamples of shredded carrots (10g each) were removed for sugar and total soluble phenolic assays.

Package headspace analyses

Oxygen, carbon dioxide, ethanol and acetaldehyde concentrations in packages were determined. Oxygen and carbon dioxide levels were measured with a Gow Mack Model 550 GC equipped with thermal conductivity detector, and a CTR I column (Alltech Associates, Deerfield, IL). Helium was the carrier gas at 30 mL/min. The injector was 100°C, detector 150°C and the column was 25°C. Ethanol and acetaldehyde in packages were determined using flame ionization chromatography. A Hewlett Packard Model 5890A GC equipped with a 5% Carbowax 20M column (Alltech Associates, Deerfield, IL) was used. Helium was the carrier gas at 20mL/min. The injector was 150°C and detector 175°C, and the column was 90°C. External standards were used for quantification of O_2, CO_2, acetaldehyde and ethanol.

Carrot quality analyses

Color measurements were made using a Hunter Labscan Model LS-5100 colorimeter. The L*, a*, and b* data were transformed to a whiteness index score (WI) using the equation $100 - [(100 - L^*)^2 + a^{*2} + b^{*2}]^{0.5}$. Sugars were extracted in cold water (10 g/20 mL) and measured by HPLC as previously described (Howard et al., 1994b). Sugars were quantified using external standards. Total soluble phenolics were extracted in 80% ethanol (10 g/20 mL) and measured using Folin-Ciocalteau reagent (Swain and Hillis, 1959). Gallic acid was used as standard.

Sensory analysis

Sensory properties were evaluated by 10 experienced judges at room temperature (25 ± 1°C) under normal room fluorescent light. Judges were served three mini-peeled carrots in three-digit randomly coded containers. Judges evaluated all samples in duplicate. Samples were expectorated and judges were given distilled water and crackers to cleanse their palates between samples. A structured 10-point scale was used with 0 = none, 10 = intense. Taste evaluation was discontinued after 3 wk storage due to excessive microbial contamination.

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Microbial analysis

Total aerobic plate count (APC), yeast, mold and lactobacilli were enumerated at each sampling time. Samples were prepared by removing 25g of product and blending for 2 min with Butterfield's dilution water. Homogenate was then diluted serially in Butterfield's dilution water to obtain dilutions for counting (Swanson et al., 1992). Samples were analyzed by pour plate method for APC incubated at 35°C for 2 days (Swanson et al., 1992), yeast and mold by the antibiotic pour plate method incubated at 25°C for 5 days (Mislivec et al., 1992), and lactobacilli by the pour plate method using APT agar incubated anaerobically at 35°C for 3 days (Vedamuthu et al., 1992). Microbiological analyses were conducted at Silliker Laboratory, College Station, TX. Microbiological testing was discontinued after 3 wk storage due to excessive microbial counts (APC $> 10^6$).

Statistical analysis

Data were statistically examined by analysis of variance ($P < 0.05$), and means were separated by Duncan's multiple range test.

RESULTS & DISCUSSION

EDIBLE COATING TREATMENT effectively retarded surface discoloration on mini-peeled carrots as determined instrumentally by Hunter color, or by trained sensory panelists (Fig. 1; Table 1). All application rates resulted in significantly reduced rates of surface discoloration relative to non-coated carrots. Retardation of surface discoloration was affected by coating application rate. Carrots treated with the highest rate (0.87 L/min) had the least surface discoloration, while those with the lowest rate (0.23 L/min) had much less surface discoloration than non-coated carrots. Hunter L-values were also affected by coating treatment (Table 1). Carrots treated with edible coating had lower L-values than non-coated carrots. Higher L-values indicate greater whiteness. No differences in Hunter a or b values occurred between treatments. Hunter a values indicate red (+) to green (−) coloration, whereas b values indicate yellow (+) to blue (−) coloration.

Coating treatments had minor effects on levels of oxygen, carbon dioxide and ethanol in the package headspace (Table 1). Carrots treated with the highest application rate (0.87 L/min) had the greatest levels of CO_2, but oxygen levels were similar to noncoated carrots. Minor differences in ethanol levels in packages were also observed between treatments. These results indicated that coating treatments did not induce or potentiate anaerobic respiration. Equilibrated levels of oxygen in the packages ranged from 8.4–13.2%, while CO_2 concentrations ranged from 8.7–10.7%. The wide variations in equilibrated levels of oxygen may indicate leaky package seams.

Coating treatments had a minor effect on sugar content. Carrots treated with the lowest rate had a higher total sugar content than non-coated carrots or those treated at the highest rate. Carrots treated with the highest rate had the lowest sucrose and total sugar content. Lower values for carrots treated with the highest application rate probably reflect a dilution effect since concentrations were expressed on a fresh weight basis. Sucrose was the predominant sugar in the Caro-Pak cultivar (2.4–3.5%), followed by similar concentrations of glucose (0.6–1.1%), and fructose (0.6–1.5%). Noncoated carrots had lower levels of glucose than polymer treated carrots. Noncoated carrots also had lower levels of fructose than those treated with 0.23 L/min. Greater glucose and fructose loss in non-coated samples may be due to greater respiratory activity. The coating may have created an internal modified atmosphere that retarded respiratory activity and glucose and fructose consumption in treated samples. Sugar concentrations were similar to levels reported by Simon (1985). Sugars are an important flavor constituent in both fresh and processed carrots (Simon, 1985; Howard et al., 1994c).

Edible coating treatment had a minor effect on levels of total soluble phenolics. Reduced levels of phenolics in carrots treated at the highest rate probably reflected a dilution effect by the polymer coating rather than metabolic changes in phenolics.

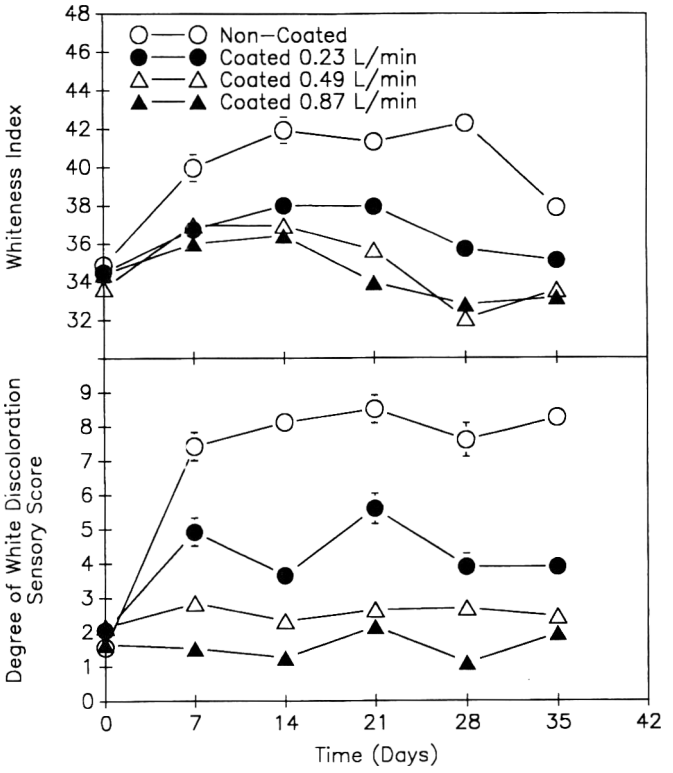


Fig. 1—White surface discoloration on mini-peeled carrots as affected by edible coating treatment and storage at 2°C. Bars represent standard error of the mean (lower than symbol size when omitted).

Phenolics affect bitterness of fresh carrots (Simon, 1985), and may be important in lignin formation on abraded carrot surfaces (Bolin and Huxsoll, 1991; Howard and Griffin, 1993).

Edible coating treatment did not affect microbial quality of the product (Table 2). No significant differences in APC, yeast, mold or lactobacilli were observed between coated and non-coated samples. Lactic acid bacteria have typically proliferated on vegetables stored in modified atmospheres (Sinell, 1980; Brackett, 1987; Carlin et al., 1990; Chervin and Boisseau, 1994). Lactics increased readily during storage, but were not affected by coating treatment. Lactobacilli increased two log cycles during 3 wk storage (3.6–5.7 log/CFU), while total aerobic counts increased almost three log cycles (3.9–6.6 log/CFU). Microbiological analysis was discontinued after 3 wk due to excessive microbial growth. These results indicated that the product shelf life was ≈3 wk. Toxic substances may be produced when microbiological counts exceed 10^6 CFU/g. The high relative humidity imparted by the coating apparently did not promote microbial growth. This was unexpected, since high relative humidity in packages typically favors the growth (Brackett, 1987). The aseptic condition of the product and an acidulant in the formulation may have suppressed microbial growth.

Edible coating treatment had a beneficial effect on sensory properties (Table 3). In addition to retarding white discoloration, coating treatments resulted in higher sensory scores for orange color intensity, fresh carrot aroma, fresh carrot flavor, and overall acceptability than non-coated carrots. As expected, orange color intensity scores were inversely related to white discoloration scores. Higher fresh carrot aroma and flavor scores may indicate the coating retarded losses of flavor volatiles from abraded carrot surfaces. We observed a large loss of volatile terpenoids from mini-peeled carrots during storage at 2°C (Howard and Dewi, 1994; unpublished data). Fresh carrot flavor and aroma scores declined during storage which indicated loss of volatile constituents. Fresh and processed carrot flavor are affected by terpenoid content (Heatherbell et al., 1971; Simon, 1985; Howard et al., 1995). Also panelists may have been biased

Table 1—Effects of edible coating on chemical composition of mini-peeled carrots stored at 2°C[a]

Treatment	Whiteness Index	Hunter L-Value	Oxygen	Carbon Dioxide	Ethanol	Sucrose	Glucose	Fructose	Total Sugar	Total Phenolics
			%		ppm		g/100g			(μg/g)
Control	39.7[b]	47.2[b]	7.0[cd]	7.5[c]	284.3[bc]	3.11[b]	0.93[d]	0.90[c]	4.94[c]	291.3[b]
Polymer										
0.23 L/min	36.3[c]	43.4[c]	8.5[b]	8.0[c]	175.6[c]	3.10[b]	1.02[b]	1.05[b]	5.18[b]	283.7[bc]
0.49 L/min	34.8[d]	42.1[d]	6.3[d]	7.7[c]	388.4[b]	3.15[b]	0.96[c]	0.96[c]	5.07[bc]	280.1[bc]
0.89 L/min	34.5[d]	41.5[d]	7.5[bc]	9.3[b]	403.2[b]	2.78[c]	0.98[bc]	0.98[bc]	4.73[d]	249.6[c]

[a] Means from five sampling times.
[b,c,d] Mean separation by Duncan's multiple range test ($P < 0.05$); values within column with similar letters not significantly different.

Table 2—Effects of edible coating on microbiological composition of mini-peeled carrots stored at 2°C[a]

Treatment	APC	Yeast	Mold	Lactobacilli
	log (CFU/g)			
Control	5.53[bc]	3.55[b]	1.95[b]	5.22[b]
Polymer				
0.23 L/min	5.44[c]	3.68[b]	1.89[b]	5.15[b]
0.49 L/min	5.91[b]	3.99[b]	1.87[b]	5.24[b]
0.89 L/min	5.68[bc]	3.83[b]	1.92[b]	4.69[b]

[a] Means from five sampling times.
[b,c] Mean separation by Duncan's multiple range test ($P < 0.05$); values within column with similar letters not significantly different.

Table 3—Effects of edible coating on sensory attributes of mini-peeled carrots stored at 2°C[a]

Treatment	White Discoloration	Orange color Intensity	Fresh carrot Aroma	Fresh carrot Flavor	Slipperiness	Overall acceptability
Control	6.91[b]	4.32[e]	4.12[c]	5.43[c]	1.87[e]	4.70[d]
Polymer						
0.23 L/min	4.00[c]	6.34[d]	5.35[b]	7.00[b]	2.53[d]	6.16[bc]
0.49 L/min	2.52[d]	7.19[c]	5.49[b]	6.92[b]	4.02[c]	6.43[b]
0.89 L/min	1.60[e]	8.20[b]	5.56[b]	6.50[b]	6.86[b]	5.53[c]

[a] Means from five sampling times.
[b-e] Mean separation by Duncan's multiple range test ($P < 0.05$); values within column with similar letters not significantly different.

by the carrot appearance. Coated carrots may have been perceived as fresher than non-coated carrots, influencing panelists to rank them higher in fresh flavor and aroma. Further studies are needed to determine if the coating retards loss of volatile flavor compounds. Degree of slipperiness was affected by application rate. Carrots treated with the highest application rate had the highest "slipperiness" scores. Several panelists indicated that the highest rate was much too slippery for commercial practice. Application rates of 0.23 and 0.49 L/min provided adequate protection against surface discoloration without producing high slipperiness. Carrots treated with edible coating had higher acceptability scores than noncoated carrots. Samples treated with the highest rate (0.87 L/min) were least preferred due to the high degree of slipperiness.

CONCLUSIONS

EDIBLE COATING TREATMENT effectively retarded surface discoloration without diminishing microbial or chemical quality of mini-peeled carrots. Sensory scores for fresh carrot flavor, fresh carrot aroma and overall acceptability were higher for coated carrots. An application rate of 0.23–0.49 L/min appeared adequate for protection against surface discoloration and for retention of flavor.

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Sensory Attributes and Instrumental Analysis Relationships for Strained Processed Carrot Flavor

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ABSTRACT

Regression models relating sensory attributes and composition of ten genotypes of processed strained carrots were developed. Fresh carrot-flavor, aroma, and aftertaste were associated with high total sugar to terpinolene ratios. Sweet taste and sweet aftertaste were associated with high levels of total sugar and high total sugar to terpinolene ratios. Cooked flavor and cooked aftertaste were associated with high terpinolene content. Selection of genotypes with high total sugar to terpinolene ratios should improve strained processed carrot sensory quality.

Key Words: strained-carrots, flavor, aroma, sugar, terpenoids

INTRODUCTION

MANY STUDIES HAVE FOCUSED on factors affecting fresh carrot quality (reviewed by Simon, 1985) but few have examined factors affecting the sensory quality of processed carrots. Raw carrot flavor is affected by free sugar content (Rygg, 1945; Alabran and Mabrouk, 1973), volatile constituents (Buttery et al., 1968, 1979; Heatherbell et al., 1971; Heatherbell and Wrolstad, 1971; and Simon et al., 1980a,b,c), bitter compounds (Carlton et al., 1961; Sarkar and Phan, 1974, 1979), and free amino acids (Alabran and Mabrouk, 1973). Harsh turpentine flavor is associated with elevated levels of terpenoids, and reduced sugar content, while sweetness and overall preference are related to high sugar content and reduced volatile components (Simon et al., 1980a,b).

Fresh carrot flavor is believed to be influenced by genetics, with a high degree of sweetness and a low level of harsh flavor being dominant (Simon, 1982,1984; Simon et al., 1982). Terpinolene and caryophyllene are usually the most abundant volatile terpenoids in fresh carrots (Heatherbell and Wrolstad, 1971b; Simon et al., 1980b,c).

Processed carrot volatiles have been characterized by a substantial loss of "higher boiling" compounds and an increase in "lower boiling" compounds (Heatherbell et al., 1971). Ethanethiol, dimethyl sulfide, acetaldehyde, octanal, and 2-decenal contribute to canned carrot flavor (Heatherbell et al., 1971). Bitterness is also known to affect carrot flavor. Isocoumarin, 6-methoxy mellein, is synthesized during storage when carrots are exposed to ethylene and is bitter (Carlton et al., 1961; Sarkar and Phan, 1974). Other phenolics such as eugenin, isochlorogenic acid, and hydroxybenzoic acid may affect bitterness (Sarkar and Phan, 1979; Maga and Lorenz, 1973). Ethylene exposure in carrots does not affect synthesis of volatile terpenoids (Simon, 1984).

Since fresh carrot flavor is affected by genetic variation, our objective was to determine the effects of genotype on processed strained carrot flavor. Relationships between sensory attributes and physicochemical variables were determined in order to identify important flavor compounds. Such data could help plant breeders screen breeding lines to improve carrot flavor.

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MATERIALS & METHODS

Materials and processing

Carrots (*Daucus carota* L.) were grown at the Texas A&M Experiment Station at Weslaco, TX in a sandy, loam soil. All genotypes were planted simultaneously and received the same water and fertilization treatments. Genotypes included five commercial cultivars; Danvers 126 (Danvers type), Kuronan (Tropical Nantes type), Lieberno (Burlicum Nantes type), Luxor (European Nantes type), Texas Goldspike (Imperator type) and 5 experimental hybrids; XPH 3738, XPH 3739, XPH 3750 and XPH 3909 (all Danvers types) and XPH 3474 (Tropical Nantes type). Twenty roots from each genotype were washed, hand peeled, blended with an equal weight of water and filled in 138 mL glass jars (24 jars per genotype). The jars were exhausted at 105°C for 2–3 min, capped and heated at 121°C for 30 min in a rotary retort at 19 rpm (Stock Pilot-Rotor 900, Stock America Inc., Milwaukee, WI).

Chemical analyses

Refractive index, total soluble phenolics, sucrose, glucose, and fructose, malic, citric and succinic acid, iso-coumarin, and volatile terpenoids were measured. Refractive index of strained carrots was measured with an Abbe refractometer. Total soluble phenolics in an 80% ethanol extract (5g/20 mL) were measured using the procedure of Swain and Hillis (1959).

Sucrose, glucose and fructose levels were measured by high performance liquid chromatography (HPLC). Sugars were extracted from strained carrots (5 g) with 80% ethanol (20 mL) using a Tekmar Model-TP 18 Tissuemizer (Cincinnati, OH). After filtering through Miracloth (Calbiochem, San Diego, CA), residues were re-homogenized twice with 20 mL of 80% ethanol, and refiltered. Filtrates were pooled and adjusted to 100 mL in volumetric flasks, using deionized water. Interfering compounds were removed by passing 10 mL aliquots through 4 cm columns containing 1g of Bio-Rex 5, 200–400 mesh anion exchange resin (Bio-Rad, Richmond, CA). Samples were filtered through 0.45 µm filters and injected into a Spectra Physics Model 2000 HPLC system fitted with a refractive index detector (Shodex Model RI-71), and a 300 × 7.8 mm Aminex HPX-87C (Bio-Rad, Richmond, CA) carbohydrate column. The mobile phase was Milli-Q water at 0.6 mL/min, and the column temperature was maintained at 85°C. Sucrose, glucose and fructose were quantified using external standards.

Malic, citric and succinic acid levels were measured by HPLC. The extraction process for organic acids was similar to that for sugars, except cold (< 4°C) deionized water was used as extraction solvent and samples were not passed through an ion-exchange column. After filtering through 0.45 µm filters, samples were injected into a Spectra Physics Model 2000 HPLC equipped with a UV-VIS detector set at 210 nm, and a 100 × 7.8 mm Fast Acid analysis column (Bio-Rad, Richmond, CA). The mobile phase was 0.001 M H_2SO_4 at 1.2 mL/min. Citric, malic and succinic acids were quantified using external standards.

Isocoumarin was isolated and characterized as described by Marinelli et al. (1990). Filtered samples were analyzed by reverse phase HPLC on a 150 × 3.9 mm, C_{18}-silica gel column (Nova-Pak, Waters, Milford, MA) using the following conditions: flow 1 mL/min; solvent program: linear gradient from 40-60% MeOH in H_2O during 10 min and then a linear gradient from 60-80% MeOH in H_2O during 15 min. UV detection was at 267 nm.

Volatiles were quantitatively determined according to the method of Simon et al. (1980C), using Tenax porous polymer traps. Analysis was performed on an Hewlett Packard Model 5890A GC equipped with an HP-1 (Hewlett Packard, Wilmington, DE), methyl silicone gum megabore column (10m × 0.53 mm) and a flame ionization detector. Helium was carrier gas at 5 mL/min and make up gas at 20 mL/min. Injector temperature was 250°C and detector 275°C. Column temperature was

Table 1—Sensory attributes evaluated with references and definitions

Sensory attributes	Reference or definitions
Aroma, flavor and aftertaste	
Fresh carrot	The aroma, flavor or aftertaste of fresh raw peeled carrots (BunnyLuv Brand Fresh Carrots, Schnucks, St. Louis, MO)
Carrot peel	The aroma, flavor or aftertaste of the peels of fresh raw carrots (BunnyLuv Brand Fresh Carrots, Schnucks, St. Louis, MO)
Immature/unripe	The aroma, flavor or aftertaste of fresh carrot tops (Carrots purchased fresh, no brand name, Schnucks, St. Louis, MO)
Cooked carrot	The aroma, flavor or aftertaste of canned carrots (Schnucks Brand Canned Carrots, no salt added, Schnucks, St. Louis, MO)
	The aroma, flavor or aftertaste of fresh boiled carrots, (Schnucks, St. Louis, MO)
Cooked sweet potatoes	The aroma, flavor or aftertaste of canned sweet potatoes (Showboat Brand Sweet Potatoes in light syrup)
Taste	
Sweet	Sucrose solution (5% w/v)
Bitter	Caffeine solution (0.05% w/v)
Salty	Sodium chloride solution (0.15% w/v)

held at 60°C for 15 min, programmed to 220°C at 3°C/min, and held at 220°C for 5 min. Volatiles were quantified using external standards.

Sensory evaluation

Sensory properties of processed strained carrot aroma, flavor, taste, and aftertaste were evaluated by 10 trained judges (two women and eight men, aged 25 to 43 yr). The judges participated in five training sessions. During the first session they were presented the entire set of samples and asked to generate descriptors which described differences among samples. During subsequent sessions the judges discussed the descriptors and defined reference standards to anchor descriptors. Through refinement of the references the judges created a consensus set of descriptors for differences among samples. The reference standards were available to the judges during each evaluation session. Evaluations were carried out in individual booths under incandescent light at room temperature (25 ± 1°C). Panelists were served 12.5 mL samples in 3-digit randomly coded 50 mL containers. Samples were served at room temperature (25 ± 1°C). All samples were served in random order. Plastic teaspoons were provided. Distilled water and expectoration cups provided. Panelists were instructed to rinse their mouths between samples. All samples were expectorated. Table 1 lists sensory attributes scored as well as reference standards used. An unstructured line scale was used with 0 cm = none, 18 cm = intense. All panelists evaluated all samples in quadruplicate.

Statistical analyses

Chemical and sensory data represent means of 4 subsamples with each jar of strained carrots serving as a subsample. Chemical and sensory data were analyzed by analysis of variance (SAS Institute, Inc., 1985) and mean separation was conducted using Duncan's multiple range test ($P<0.05$). Regression techniques were used to estimate relationships between independent (chemical) variables and response variables (sensory) using stepwise multiple linear regression procedures (SAS Institute, Inc., 1985).

RESULTS & DISCUSSION

Sensory results

Aroma, flavor, taste and aftertaste sensory attributes of strained carrots were related to genetic variation (Tables 2 and 3). Luxor, XPH 3474, and XPH 3750 consistently had higher scores for desirable fresh attributes, fresh carrot aroma, flavor, and aftertaste than other genotypes. These genotypes had low scores for undesirable cooked flavor and cooked aroma attributes. Danvers 126, Kuronan, Lieberno and XPH 3909 had low scores for fresh carrot attributes. Texas Goldspike had exceptionally high scores for sweet taste and sweet aftertaste, but

Table 2—Aroma and flavor descriptors for strained carrots as related to genotype

Genotype	Cooked carrot aroma[a]	Fresh carrot aroma	Cooked sweet potato aroma	Carrot peel aroma	Immature aroma	Cooked carrot flavor[b]	Fresh carrot flavor	Cooked sweet potato flavor	Carrot peel flavor	Immature flavor
Kuronan	7.2 a[c]	2.8 c	7.0 ab	1.5 bcd	1.8 abcd	7.5 a	3.4 d	5.6 a	1.8 cde	2.8 a
Lieberno	6.8 a	3.4 c	8.1 a	1.4 cd	1.6 abcd	6.5 ab	3.3 d	5.6 a	2.1 bcde	2.6 a
Luxor	5.3 bc	7.6 a	3.6 e	1.4 cd	1.3 bcd	4.9 cd	9.3 a	2.5 b	1.3 e	1.3 c
XPH 3474	4.2 cd	7.2 ab	4.6 de	1.4 cd	1.2 cd	3.5 d	8.0 ab	3.0 b	2.4 abc	2.6 a
XPH 3738	7.0 a	3.9 c	5.6 bcd	2.2 ab	2.0 abc	7.4 a	4.0 cd	4.7 a	2.4 abc	2.4 ab
XPH 3739	7.3 a	4.1 c	4.8 cde	2.0 abc	2.1 ab	7.2 a	4.5 cd	3.2 b	2.3 abcd	3.0 a
XPH 3750	6.0 ab	5.8 b	4.0 e	2.2 ab	2.0 abc	5.3 bc	6.7 b	2.3 b	2.8 ab	2.4 ab
Texas Goldspike	6.8 a	4.1 c	6.2 bc	1.6 bcd	1.4 bcd	7.2 a	5.0 c	5.4 a	1.5 de	1.5 bc
XPH 3909	6.3 ab	3.6 c	5.6 bcd	2.5 a	2.4 a	6.5 ab	3.6 cd	5.4 a	2.9 a	2.5 a
Danvers 126	6.5 ab	3.1 c	7.7 a	1.2 d	1.0 d	7.2 a	3.9 cd	4.8 a	2.1 bcde	2.1 abc

[a] 0 = no aroma; 18 = intense aroma.
[b] 0 = no flavor; 18 = intense flavor.
[c] Values within a column with the same letters are not significantly different (LSD, $P<0.05$).

Table 3—Taste and aftertaste descriptors for strained carrots as related to genotype

Genotype	Sweet taste[a]	Bitter taste	Salty taste	Cooked carrot after-taste[b]	Fresh carrot after-taste	Cooked sweet potato after-taste	Carrot peel after-taste	Immature after-taste
Kuronan	7.2 bc[c]	2.7 ab	1.4 bc	6.0 a	2.7 b	4.9 a	2.0 bc	2.3 a
Lieberno	6.2 cde	2.1 bcd	2.0 ab	5.8 ab	2.9 b	4.9 a	1.7 abc	1.9 abc
Luxor	7.8 ab	1.4 d	1.6 abc	4.1 cd	7.4 a	1.6 d	1.3 c	1.2 c
XPH 3474	7.6 ab	2.0 bcd	1.2 c	3.3 d	7.1 a	1.9 cd	2.1 abc	2.1 ab
XPH 3738	6.0 de	2.2 abc	1.5 bc	6.0 a	3.3 b	4.6 a	2.0 abc	2.1 ab
XPH 3739	5.3 e	2.7 ab	2.2 a	6.2 a	3.6 b	3.0 bc	1.9 abc	1.8 abc
XPH 3750	6.1 cde	2.2 abc	1.6 abc	4.5 bcd	6.6 a	2.0 cd	2.3 ab	2.1 ab
Texas Goldspike	8.4 a	1.6 cd	1.4 bc	5.9 a	3.9 b	4.5 a	1.5 bc	1.4 bc
XPH 3909	6.9 bcd	2.2 abc	1.8 ab	5.4 abc	3.4 b	4.3 a	2.4 a	2.0 ab
Danvers 126	6.0 de	3.1 a	1.2 c	6.1 a	3.2 b	3.9 ab	1.9 abc	2.0 ab

[a] 0 = no taste; 18 = intense taste.
[b] 0 = no aftertaste; 18 = intense aftertaste.
[c] Values within a column with the same letters are not significantly different (LSD, $P<0.05$).

Table 4—Chemical factors and soluble phenolic content of strained carrots as related to genotype

Genotype	Sucrose	Glucose	Fructose	Total sugar	°Brix	Malic acid	C tric acid	Succinic acid	Total acid	Sugar: acid	Total soluble phenolics
	g/100g										µg/g
Kuronan	1.67 bc[a]	0.45 ab	0.38 ab	2.51 bc	5.93 b	0.287 b	0.072 b	0.078 a	0.438 ab	5.78 ab	254.8 b
Lieberno	1.18 d	0.44 ab	0.31 b	1.93 e	4.97 de	0.283 bc	0.062 bc	0.065 b	0.424 b	4.54 c	224.7 c
Luxor	1.47 cdb	0.51 ab	0.40 ab	2.38 b	5.80 bc	0.270 bc	0.055 c	0.065 b	0.390 b	6.10 ab	197.1 d
XPH 3474	1.26 d	0.61 a	0.46 a	2.33 bcde	5.03 cde	0.274 bc	0.062 bc	0.084 a	0.420 bc	5.54 b	231.6 c
XPH 3738	1.29 d	0.44 ab	0.37 ab	2.10 de	5.20 bcde	0.322 ab	0.068 bc	0.085 a	0.475 ab	4.43 c	244.1 bc
XPH 3739	1.62 bc	0.38 b	0.31 b	2.30 bcde	5.37 bcde	0.314 ab	0.080 ab	0.074 ab	0.468 ab	4.93 bc	234.8 c
XPH 3750	1.19 d	0.60 a	0.46 a	2.24 cde	4.80 e	0.232 c	0.055 c	0.056 b	0.344 c	6.51 a	183.9 e
Texas Goldspike	2.45 a	0.36 b	0.32 b	3.13 a	6.90 a	0.335 ab	0.087 a	0.079 a	0.502 a	6.24 ab	274.5 a
XPH 3909	1.88 b	0.40 b	0.36 b	2.65 b	5.70 bcd	0.330 ab	0.064 bc	0.081 a	0.474 ab	5.58 b	232.1 c
Danvers 126	1.20 d	0.47 ab	0.40 ab	2.07 de	5.63 bcd	0.342 a	0.071 b	0.074 ab	0.487 ab	4.31 c	247.2 bc

[a] Values within a column with the same letters are not significantly different (Duncan's multiple range test, $P<0.05$).

Table 5—Volatile terpenoid levels (500 × ppm) in strained carrots

Genotype	α-Pinene	β-Pinene	Myrcene	α-Phellandrene	α-Terpinene	Limonene	γ-Terpinene	Terpinolene	Bornyl Acetate	Caryophyllene	Total
Danvers 126	57 b[a]	15 c	92 a	4 b	25 b	34 b	148 a	233 abc	28 b	175 bc	811 a
Kuronan	51 b	17 c	69 b	12 a	16 c	44 a	149 a	263 a	26 b	113 cd	760 a
Lieberno	5 e	ND	10 e	ND	8 cdef	12 d	37 de	123 d	ND	228 ab	423 bc
Luxor	35 c	5 e	37 de	ND	2 f	13 cd	23 e	65 e	ND	121 bcd	301 c
Texas Goldspike	5 e	ND	18 de	2 b	4 ef	20 cd	58 bcde	245 ab	ND	50 d	402 bc
XPH 3474	158 a	135 a	44 c	ND	6 def	50 a	85 de	85 de	118 a	61 d	742 a
XPH 3738	16 de	11 d	31 cd	7 b	14 cd	22 c	200 bcde	200 c	ND	322 a	676 a
XPH 3739	28 cd	40 b	20 de	8 ab	12 cde	22 c	205 cde	205 bc	ND	148 bcd	528 b
XPH 3750	26 cd	4 ef	23 de	ND	80 a	15 cd	103 bc	103 de	5 c	192 bc	522 b
XPH 3909	19 d	1 f	33 cd	3 b	5 def	22 cd	203 bcde	203 bc	ND	117 bcd	455 b

[a] Values within a column with the same letters are not significantly different (Duncan's multiple range test, $P<0.05$).

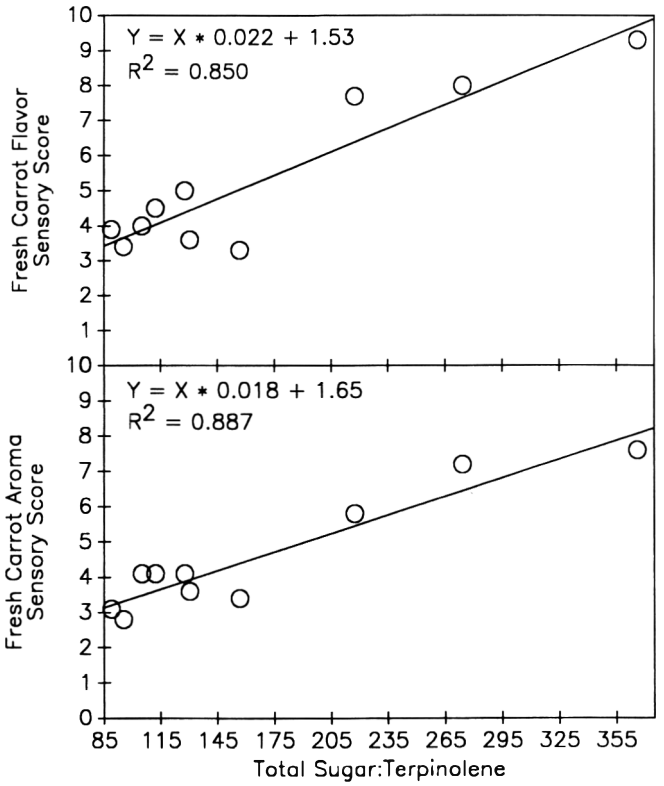


Fig. 1—Regression analysis of fresh carrot flavor and aroma vs total sugar (ppm) to terpinolene ratio (ppm × 500) in strained carrots.

received high scores for cooked attributes, cooked carrot aroma, cooked carrot flavor, cooked sweet potato flavor, cooked carrot aftertaste, and cooked sweet potato aftertaste. Danvers 126, XPH 3739, and XPH 3738 had low scores for sweet taste and sweet aftertaste. All genotypes had low scores for bitter, salty, immature, and carrot peel attributes. The low flavor intensity scores for these attributes indicated they were of minor importance in flavor perception. Conversely, high intensity scores for fresh, cooked and sweet attributes indicated they were highly associated with strained carrot flavor. Low sensory scores for Danvers 126 for fresh carrot flavor and fresh carrot aroma was unexpected since the genotype is widely used in the carrot processing industry. Results indicate that new genotypes had more intense fresh flavor characteristics.

Instrumental results

Sugar, organic acid, and soluble phenolics, and sugar-to-acid ratios of strained carrots were related to genotype (Table 4). Sucrose was the predominant sugar in strained carrots (1.18–2.45 g/100g), followed by glucose (0.36–0.61 g/100g), and fructose (0.31–0.46 g/100g). Total sugar content ranged from 1.93–3.13 g/100g. Expressed on fresh carrot weight instead of strained carrot % weight (strained carrots were diluted twofold), total sugar content ranged from 3.70–6.64. Simon (1985) reported total sugar content in fresh carrots ranged from 3 to 8% with sucrose predominant. Texas Goldspike and XPH 3909 had exceptionally high levels of total sugars, while Danvers 126 a popular processing carrot had low levels. Brix readings followed the same trend as total sugars, but concentrations were about 2-fold higher. Apparently, soluble carbohydrates in addition to sucrose, glucose and fructose were present in carrots that influenced soluble solids concentration. Total sugar concentrations correlated better ($R=0.72$) with sweet flavor scores than did °brix readings ($R=0.65$).

Malic was the predominant organic acid (0.232-0.342 g/100g), followed by succinic (0.056–0.085 g/100g), and citric (0.055-0.087 g/100g). Texas Goldspike and Danvers 126 had high levels of organic acids, while Luxor and XPH 3750 had low levels. Sugar to acid ratios ranged from 4.31 to 6.51. XPH 3750, Texas Goldspike and Luxor had high sugar to acid ratios, while XPH 3738 and Lieberno had low ratios. Total soluble phenolic concentration ranged from 184–275 µg/g. Texas Goldspike, Kuronan, and Danvers 126 had high levels of soluble phenolics, while Luxor and XPH 3750 had low levels. Phenolic compounds, especially isocoumarin affect bitterness of fresh carrots (Simon, 1985). No isocoumarin was detected in proc-

Table 6—Stepwise multiple linear regression models between sensory attributes and objective measurements in strained carrots

Sensory attribute	Regression model
Cooked carrot flavor	= 3.17 + 0.010 * terpinolene (r^2=0.63, p=0.0103)
Cooked sweet potato flavor	= 0.63 + 0.010 * terpinolene (r^2=0.74, p=0.0028)
Carrot peel flavor	= 5.88 − 0.015 * total phenolics (r^2=0.68, p=0.0063)
Cooked carrot aroma	= 11.54 − 12.01 * glucose + 0.012 * alpha terpinene (r^2=0.83, p=0.0022)
Sweet taste	= −7.61 + 7.85 * total sugar − 0.19 * total sugar:terpinolene (r^2=0.97, p=0.0005)
Sweet after-taste	= −5.18 + 5.88 * total sugar − 0.14 * total sugar:terpinolene (r^2=0.90, p=0.0009)
Fresh carrot after-taste	= 0.72 + 0.043 * total sugar:terpinolene (r^2=0.96, p=0.0001)
Cooked carrot after-taste	= 1.99 + 0.009 * terpinolene (r^2=0.76, p=0.0023)
Cooked sweet potato after-taste	= 0.210 + 0.0098 * terpinolene (r^2=0.83, p=0.0006)
Bitter taste	= 1.58 + 0.001 * total terpinoids (r^2=0.45, p=0.048)

essed samples (data not shown). The HPLC detection limit for isocoumarin was 1 ppm. Isocoumarin is synthesized in carrots in response to ethylene or abiotic stress (Carlton et al., 1961; Sarkar and Phan, 1979). Our results indicated isocoumarin did not affect bitterness of strained carrots if fresh carrots were not exposed to ethylene or abiotic stress prior to processing.

Terpinolene, caryophyllene, and gamma terpinene were major terpenoids in strained carrots accounting for 31%, 27% and 13% of total terpenoids (Table 5). Relative amounts of volatile terpenoids were similar to those previously reported (Heatherbell et al., 1971; Simon et al., 1980a,b,c). Terpenoid content varied greatly among genotypes. Danvers 126, Kuronan, XPH 3738, XPH 3739, and XPH 3474 had high levels of terpenoids while Luxor had low levels. XPH 3474 was unique in that it contained high levels of α and β pinene, and bornyl acetate. The wide variation in terpenoid content in genotypes grown under identical environmental conditions indicated genetic factors were responsible. Dominance for low quantities of carrot volatile terpenoids has been observed in F_1 hybrids (Simon, 1982), which indicated potential for breeding genotypes with low volatile content.

Relationships between sensory and instrumental measurements

Regression models between sensory and instrumental measurements of strained carrot flavor were determined to establish relationships between data sets. Fresh carrot flavor and fresh carrot aroma were associated with high total sugar (sum of sucrose, glucose and fructose) to terpinolene ratios (Fig. 1). Both models fit the data well with R^2 values of 0.85 and 0.89, respectively. High sugar levels and low levels of terpinoids are important factors affecting fresh carrot flavor (Simon et al., 1980b; Simon, 1985).

Other important regression models were developed (Table 6). Terpinolene concentration was associated with several sensory flavor attributes. High levels of terpinolene were negatively associated with fresh flavor, fresh aroma, fresh after-taste and sweet taste of strained carrots. Conversely, terpinolene levels were positively associated with cooked carrot flavor. 2-nonenal is also reported to impart cooked flavor in carrots (Buttery et al., 1968).

Harsh flavor in fresh carrots was associated with elevated levels of volatile terpenoids, including terpinolene (Simon et al., 1982). Cooked carrot aroma was negatively associated with glucose content. Low sugar levels and high cooked carrot aroma scores may be due to reaction of glucose in Maillard and/or carmelization reactions during thermal processing (Hodge, 1953; O'Brien and Morrissey, 1989). Maillard reaction products affected color, flavor, and nutritional quality of processed and stored products (Hodge, 1967; O'Brien and Morrissey, 1989).

Bitter flavor sensory scores were related to total terpenoid content (R^2=0.45). The low R^2 value indicated other unknown factors contributed to bitterness. Isocoumarin was reported to cause bitterness in fresh carrots (Sarkar and Phan, 1979), but none was detected in strained carrots.

CONCLUSIONS

RELATIONSHIPS between sensory attributes and chemical components in 10 genotypes of strained processed carrots were developed. Fresh carrot flavor, aroma and aftertaste were associated with high total sugar to terpinolene ratios. Cooked flavor and aftertaste attributes were associated with elevated levels of terpinolene. Results indicated plant breeder selection for genotypes with high sugar content and reduced levels of volatile terpenoids, especially terpinolene should improve strained carrot flavor.

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Phytate Reduction in Brown Beans (*Phaseolus vulgaris* L.)

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ABSTRACT

Brown beans (*Phaseolus vulgaris* L.) were subjected to treatments to evaluate effects of pH, temperature, $CaCl_2$, tannase and fermentation on degradation of phytate. Soaking was performed at 21°C, 37°C and 55°C at pH 4.0, 6.0, 6.4, 7.0, and 8.0. Optimal conditions for phytate degradation were pH 7.0 and 55°C. After soaking 4, 8 or 17 hr at these conditions 79%, 87% and 98% of phytate was degraded, respectively. Addition of tannase enhanced reduction of phytate. Fermentation of presoaked whole beans resulted in reduction of 88% of phytate after 48 hr.

Key Words: phytate, tannase, brown beans, fermentation

INTRODUCTION

MUCH OF THE WORLD POPULATION relies on legumes as staple foods particularly in combination with cereals. Legumes are often advocated in Western diets because of their beneficial nutritional effects and because they are a low cost source of protein. Use of legumes in the human diet may increase in less developed regions of the world and also in Western countries. Therefore, more information is needed about the potential nutritional implications of legume based diets.

The content of iron in brown beans (*Phaseolus vulgaris* L.) is very high (5 mg/100 g dried beans; The Swedish National Food Administration, 1986). However, the beans also contain high amounts of phytate which make the bioavailability of the iron low. The phytate molecule is negatively charged at physiological pH and chelates with several mineral elements, including iron, zinc, magnesium and calcium. This forms insoluble complexes thereby making minerals unavailable for absorption (Weingartner and Erdman, 1978). Our objective was to investigate different methods to hydrolyze phytate in brown beans (*Phaseolus vulgaris* L.).

The beans probably also contain important amounts of polyphenols. We hypothesized this because of the brown color and because other beans of the species *Phaseolus vulgaris* L. contain high amounts of polyphenols (Paradez-Lopez and Harry, 1989). Certain phenolic compounds are powerful inhibitors of iron absorption (Brune, 1989). Phytate was less degraded during fermentation of sorghum with high tannin content as compared with white sorghum (Svanberg et al. (1993)). This may indicate an inhibitory effect of polyphenols on phytase activity.

Various methods for food processing and preparation, such as soaking (Chang et al., 1977; Ologhobo 1989; Sattar et al., 1989; Iyer et al., 1980), germination (Taberkhia and Luh, 1980; Suberbie et al., 1981; Sattar et al., 1985) and fermentation (Sudarmadji and Markakis, 1977; Parades-Lopez and Harry, 1989; Marfo et al., 1990) which activate the intrinsic phytase in legumes have reduced the content of phytate. These methods have also reduced the content of polyphenols.

Phytases hydrolyze phytate to myo-inositol and inorganic phosphate via intermediate myo-inositol phosphates (penta- to monophosphates) (reviewed by Irving, 1980). In beans of *Phaseolus vulgaris* L. (e.g. Navy beans, California Small White bean, Dwarf French bean) studies have shown that phytases had pH optima at 5.2–5.3 and temperature optima at 40–60°C (Gibbins and Norris, 1963; Becker et al., 1974; Chang et al., 1977; Lolas and Markakis, 1977). Scott (1991) showed completely different pH optima. After extracting phytases from nine cultivars of *Phaseolus vulgaris* L. (e.g. Pinto beans, Navy beans, Red kidney beans). All phytases had alkaline activity, with as much as 3.5 times higher activity at pH 8.0 than at pH 5.0.

Our objective was to determine whether the phytase in brown beans had alkaline activity and any effects of soaking, addition of 1 mM $CaCl_2$ or tannase and fermentation on phytate degradation.

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MATERIALS & METHODS

Seeds and enzyme

Dried brown beans (*Phaseolus vulgaris* L.) of the 1991 crop were purchased in a local grocery and stored at room temperature (≈21°C) until used. The dried beans contained 20g protein, 1.5g fat, 56g carbohydrate and 6.5 mg iron/100 g/(dm).

Tannase was purchased from Enzyme Development Corporation (New York). The enzyme was a standardized fungal enzyme system, derived from Aspergillus oryzae, which hydrolyse tannin-like compounds. A stemsolution containing 2000 units tannase activity/mL was prepared. One unit of tannase activity was defined as the change of absorbency at 310 µm (30°C, 1 cm cell) per min using 0.004% tannic acid in 0,02M acetate buffer (pH 4.7).

Preparation of seeds

Beans were ground directly (bean flour 1) with a coffee-grinder (Kenwood) or pre-soaked 17 hr in 10 parts 37°C de-ionized water, dried in a hot air oven (Memmert Model BKE 30) at 55°C for 24 hr and then ground (bean flour 2). The presoaking water was decanted and analyzed for inositol hexa-, penta-, tetra- and triphosphates before drying.

Soaking

Duplicate samples (1 g) of ground beans (flour 1 or 2) were soaked in 10 mL soaking solution. The soaking medium was either 20 mM citrate-buffer pH 4.5 or pH 6.0, de-ionized water or 100 mM tris-buffer pH 7.0 or pH 8.0. The pH of the tris-buffer was set at the soaking temperature since the pH of this buffer solution changes with temperature. $CaCl_2$ was added to one set of samples. 9.5 mL soaking medium and 0.5 mL tannase stem solution was added to some samples. The samples were incubated in waterbath at 21°C, 37°C or 55°C for 4 hr, 8 hr or 17 hr.

Fermentation

Beans were fermented whole or ground (flour 2) without addition of a starter culture. The whole beans were pre-soaked for 17 hr in 37°C de-ionized water and then dried for 24 hr at 55°C prior to fermentation. Duplicate samples (1 g) of ground beans were soaked in 4 mL de-ionized water, covered with parafilm, placed in a 21°C shaking water bath and fermented for 24 hr. The temperature was then raised to 30°C and fermentation continued for 24 hr or 48 hr. Duplicate samples (2g) of whole beans were fermented in 8 mL water. After fermentation of whole beans, the water was decanted and analyzed for IP6-IP3. Whole fermented beans were freeze-dried, ground and analyzed.

Determination of inositol phosphates

Duplicate samples (0.5g) of bean flour 1, flour 2 or freeze-dried fermented beans were extracted with 0.5M HCl (20 mL) for 3 hr. Extracts were centrifuged and the supernatant decanted, frozen overnight, thawed and centrifuged again. For soaked samples 30 mL HCl was added to stop enzymatic activity and to extract inositol phosphates. The strength

Table 1—Soaking conditions and results when ground beans (flour 1) were soaked[a]

Soaking medium	pH	Temp (°C)	Time (hr)	IP_6[b] (μmol/g)	IP_5 (μmol/g)	IP_4 (μmol/g)	IP_3 (μmol/g)	% reduction of IP_6
H_2O	6.4	21	4	12.7 ± 0.10	1.7 ± 0.02	0.3 ± 0.02	0.3 ± 0	0
H_2O	6.4	37	4	11.8 ± 0.22	1.7 ± 0.02	0.7 ± 0.01	0.6 ± 0.02	6
H_2O	6.4	55	4	10.2 ± 0.29	2.6 ± 0.00	1.8 ± 0.06	1.5 ± 0.04	19
H_2O	6.4	37	2+					
adj. with NaOH	8.0	37	2	11.7 ± 0.35	2.5 ± 0.12	0.8 ± 0.09	0.8 ± 0.32	7
citrate-buffer	4.5	21	4	11.3 ± 0.04	0.9 ± 0.02	0.2 ± 0.02	n.d*	10
citrate-buffer	4.5	37	4	10.9 ± 0.09	1.1 ± 0	1.1 ± 1.10	0.1 ± 0.01	13
citrate-buffer	4.5	55	4	9.3 ± 0.04	2.1 ± 0.03	0.4 ± 0.01	0.3 ± 0.08	26
citrate-buffer	4.5	55	17	8.2 ± 0.63	3.1 ± 0.04	1.0 ± 0.03	0.8 ± 0.07	34
citrate-buffer	6.0	37	4	12.4 ± 0.34	1.6 ± 0.05	0.5 ± 0.04	0.5 ± 0.01	2
citrate-buffer	6.0	37	2+					
adj. with NaOH	8.0	37	2	5.9 ± 0.35	4.7 ± 0.10	2.8 ± 0.10	1.2 ± 0.05	53
tris-buffer	7.0	21	4	13.5 ± 0.11	2.0 ± 0.07	0.5 ± 0.01	0.5 ± 0.10	0
tris-buffer	7.0	37	4	10.3 ± 0.25	2.8 ± 0.06	1.3 ± 0.03	1.2 ± 0.06	18
tris-buffer	7.0	55	4	2.7 ± 0.14	1.5 ± 0.18	4.4 ± 0.04	7.8 ± 0.06	79
tris-buffer	7.0	55	8	1.7 ± 0.13	0.4 ± 0	3.9 ± 0.15	11.0 ± 0.39	87
tris-buffer	7.0	55	17	0.3 ± 0.01	0.2 ± 0.02	2.3 ± 0.12	10.4 ± 0.25	98
tris-buffer	8.0	21	4	8.1 ± 0.56	4.7 ± 0.20	2.6 ± 0.05	1.3 ± 0.02	35
tris-buffer	8.0	37	4	5.4 ± 0.03	1.1 ± 0.01	5.7 ± 0.04	5.5 ± 0.07	57
tris-buffer	8.0	55	4	6.8 ± 0.12	1.2 ± 0.02	4.8 ± 0.20	5.4 ± 0.04	46
tris-buffer + 1 mM $CaCl_2$	8.0	21	4	8.4 ± 0.19	2.8 ± 0.02	1.9 ± 0	1.3 ± 0.02	33

[a] Values are μmol/g dried sample and are given as means ± S.D. for duplicate samples.
[b] IP_6-IP_3: Inositol hexa-, penta-, tetra-, and triphosphate.
* n.d: not detectable

Table 2—Free *myo*-inositol content in soaked ground beans (flour 1)

Soaking medium	pH	Temp (°C)	Time (hr)	Free *myo*-inositol (μmol/g dry sample)
H_2O	6.4	55	17	3.1 ± 0.13
citrate-buffer	4.5	55	17	3.5 ± 0.07
tris-buffer	7.0	55	17	1.6 ± 0.06

of added HCl was adjusted depending on pH of the soaking medium to provide pH similar to that when 0.5M HCl was added to dry samples. The extraction was continued for 3 hr.

After the second centrifugation, an aliquot (15 mL) of supernatant was evaporated to dryness and then redissolved in 0.025M HCl (15 mL). Inositol phosphates were separated from the crude extract by ion exchange chromatography according to Sandberg and Ahderinne (1986). The inositol hexa-, penta-, tetra- and triphosphates were determined by ion-pair C 18 reverse-phase HPLC using formic acid/methanol and tetrabutylammoniumhydroxide in the mobile phase (Sandberg et al., 1989; Sandberg and Ahderinne, 1986). The HPLC comprised an HPLC pump (Waters Model 510, Waters Associates Inc., Massachusetts), a C 18 Chromasil (5μ) column 2 mm i.d and a refractive index detector (ERC-7510 RI-detector Erma Optical Works Ltd., Japan). Flow rate was 0.4 mL/min. Retention times and peak areas were measured by a laboratory data system, HP 3350 (Hewlett Packard Ltd.). Injections were made with a 20 μL loop.

Determination of free myo-inositol

Ethanol (55 mL) and 0.1 mg of internal standard (pentaerytritol) was added to soaked samples. They were magnetically stirred for 15 min and centrifuged. The supernatants (2 mL) were evaporated to dryness and then silylated according to Santa-Maria et al. (1985). The silylated samples were analyzed for free myo-inositol by capillary gas chromatography. Analyses were performed on a Carlo Erba HRGC-5300 HT Mega Series (Milan, Italy) fitted with an on-column injector and a FID detector (400°C). An HT polyimide-fused silica column (25 m × 0.32 i.d.) (Quartz and Silice, France) was static coated with cross-linked SE-54 (Alltech Associates, Deerfield, IL) according to the method of Blomberg et al. (1982); film thickness was 0.05 μm. Helium was carrier gas at a linear gas velocity of 60 cm/sec at 60°C. A high-capacity gas purifier (Oxisorb, Messer Griesheim, Germany) and a 5-Å molecular sieve (Alltech Associates) were used in the carrier gas line. Samples were dissolved in ethyl acetate and injected on-column at 60°C. A linear temperature program (20°C/min) was run up to 389°C. Integration was performed with a PC-pack Softran Kontron Instrument (Milan, Italy).

RESULTS

Effects of time, pH and temperature of soaking

The content of inositol hexa- and pentaphosphate in raw material was 12.6 and 1.01 μmol/g dry sample respectively, and the content of inositol tetra- and triphosphate was not detectable. The effects of various pH values and temperatures on phytate degradation in brown beans were compared (Table 1 and Table 2). When bean flour 1 (table 1) was soaked in de-ionized water at different temperatures, the greatest phytate reduction occurred at 55°C. After 8 hr incubation the pH dropped gradually from 6.4 to 4.8 (Fig. 1). Soaking in citrate-buffer pH 4.5 resulted in a small reduction ($p < 0.01$) of phytate that increased with in-

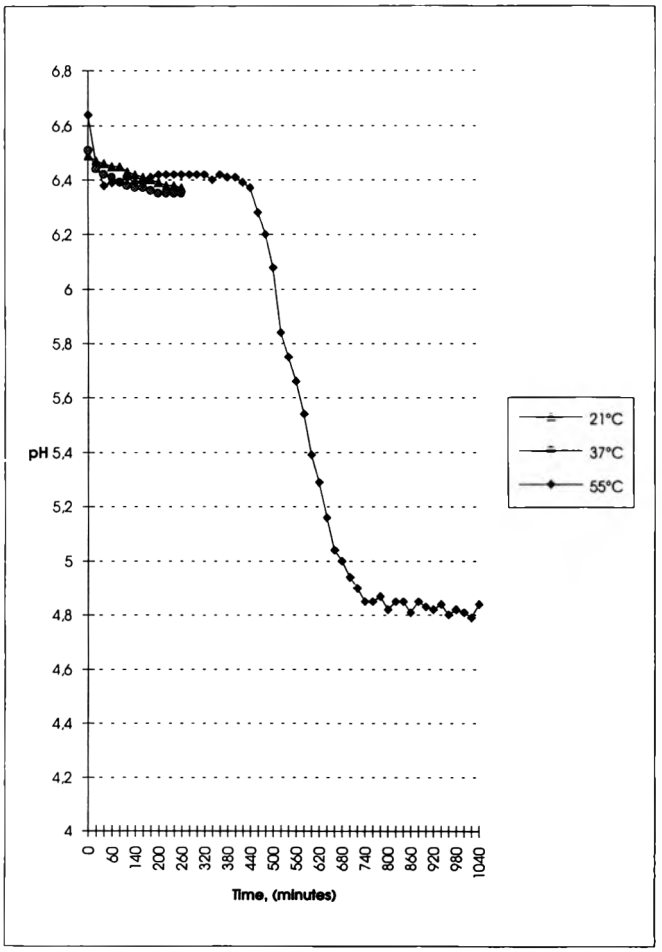


Fig. 1—pH change during soaking of flour 1 in de-ionized water as related to temperature.

Table 3—Soaking conditions and results when beans were pre-soaked in de-ionized water for 17 hr at 37°C and then dried in a hot air oven at 55°C for 24 hr, ground (flour 2) and then soaked[a]

Soaking medium	pH	Temp (°C)	Time (hr)	IP_6[b] µmol/g	IP_5 µmol/g	IP_4 µmol/g	IP_3 µmol/g	% reduction of IP_6
H_2O	6.4	21	4	5.6 ± 0.57	0.9 ± 0.04	0.2 ± 0	n.d*	55
	6.4	37	4	7.5 ± 0.37	1.0 ± 0.05	0.3 ± 0.02	0.1 ± 0.07	39
	6.4	55	4	8.5 ± 0.12	2.4 ± 0.04	1.1 ± 0.02	0.6 ± 0.06	33
	6.4	55	8	7.1 ± 0.40	1.8 ± 0.02	1.7 ± 0.02	0.8 ± 0.02	44
	6.4	55	17	1.91 ± 0.13	1.2 ± 0.04	1.9 ± 0.03	1.4 ± 0.08	85
citrate-buffer	6.0	37	4	10.4 ± 0.56	1.7 ± 0.11	0.3 ± 0.00	0.5 ± 0.40	17
tris-buffer	7.0	55	4	6.2 ± 0.45	3.3 ± 0.21	2.5 ± 0.13	2.5 ± 0.17	51
	7.0	55	8	1.6 ± 0.01	0.5 ± 0.02	3.5 ± 0.04	8.4 ± 0.61	87
	7.0	55	17	0.2 ± 0.01	0.06 ± 0.04	1.8 ± 0.67	8.7 ± 0.46	98

[a] Values are µmol/g dried sample and are given as means ± S.D. for duplicate samples.
[b] IP_6-IP_3: Inositol hexa-, penta-, tetra- and triphosphate.
* n.d: not detectable

Table 4—Soaking of ground beans (flour 1) at diferent conditions with addition of tannase[a]

Soaking medium	pH	Temp (°C)	Time (hr)	IP_6[b] µmol/g	IP_5 µmol/g	IP_4 µmol/g	IP_3 µmol/g	% reduction of IP_6
H_2O	6.4	37	4	5.7 ± 0.20	0.8 ± 0.01	0.4 ± 0.01	0.1 ± 0.03	55
citrate-buffer	6.0	37	4	12.1 ± 0.07	1.1 ± 0	0.3 ± 0.01	n.d*	4
H_2O	6.4	37	2+					
adj. with NaOH	8.0		2	11.7 ± 0.01	1.6 ± 0.01	0.5 ± 0.01	0.2 ± 0.04	7
citrate-buffer	6.0	37	2+					
adj. with NaOH	8.0		2	8.8 ± 0.06	3.1 ± 0.05	0.7 ± 0.05	n.d*	30

[a] Values are µmol/g dried sample and are given as means ± S.D. for duplicate samples.
[b] IP_6-IP_3: Inositol hexa-, penta-, tetra- and triphosphate.
* N.d: not detectable

Table 5—Soaking conditions when beans pre-soaked for 17 hr in 37°C de-ionized water, dried in a hot air oven for 24 hr and then ground (flour 2) were soaked with addition of tannase[a]

Soaking medium	pH	Temp (°C)	Time (hr)	IP_6[b] µmol/g	IP_5 µmol/g	IP_4 µmol/g	IP_3 µmol/g	% reduction of IP_6
H_2O	6.4	37	4	7.0 ± 0.50	0.9 ± 0.23	0.2 ± 0.13	0.0 ± 0.05	44
citrate-buffer	6.0	37	4	10.2 ± 0.45	1.9 ± 0.06	0.4 ± 0.01	0.2 ± 0.02	19

[a] Values are µmol/g dried sample and are given as means ± S.D. for duplicate samples.
[b] IP_6-IP_3: Inositol hexa-, penta-, tetra- and triphosphate.

Table 6—Fermentation of brown beans pre-soaked for 17 hr in 37°C de-ionized water, dried in a hot air oven for 24 hr and then ground (flour 2)[a]

Time (hr)	Temp (°C)	IP_6[b] µmol/g	IP_5 µmol/g	IP_4 µmol/g	IP_3 µmol/g	% reduction of IP_6
24	21	4.3 ± 0.30	0.7 ± 0	0.5 ± 0.05	0.3 ± 0.09	66
24+	21					
24	37	3.3 ± 0.21	1.0 ± 0.26	1.1 ± 0.35	0.6 ± 0.56	74
24+	21					
48	37	4.0 ± 0.19	1.0 ± 0.14	1.4 ± 0.13	2.1 ± 0.30	68

[a] Values are µmol/g dried sample and are given as means ± S.D. for duplicate samples.
[b] IP_6-IP_3: Inositol hexa-, penta-, tetra- and triphosphate.

creasing temperature. Soaking in tris-buffer pH 7.0 resulted in a higher degradation of phytate than soaking in pH 4.5 at 55°C ($p < 0.001$) or soaking in de-ionized water at 37°C ($p < 0.05$) or 55°C ($p < 0.001$). Soaking in tris-buffer pH 8.0 resulted in a higher degradation of phytate than soaking in pH 4.5 ($p < 0.02$) or soaking in de-ionized water ($p < 0.01$) at all temperatures. After 17 hr incubation of bean flour 1 in tris-buffer pH 7.0 at 55°C, the phytate had been degraded by 98% and the sum of inositol hexa- and pentaphosphate was 0.45 µmol/g. The content of free myo-inositol in samples soaked 17 hr at 55°C and pH 6.4, 4.5 and 7.0 was compared (Table 2). The formation of free myo-inositol was greatest at pH 4.5.

Phytate reduction was more extensive when beans were pre-soaked before ground to flour (flour 2, Table 3) when soaking in de-ionized water at all temperatures ($p < 0.01$). When flour 2 was soaked for 17 hr in pH 8.0 and at 55°C, the degradation of phytate was 98%, the same as that achieved after soaking flour 1 in the same manner.

Effect of calcium

Reduction of phytate when incubated at 21°C in tris-buffer pH 8.0 for 4h with addition of 1mM $CaCl_2$ was not significantly different than without calcium (Table 1). Release of inorganic phosphate was measured according to Fiske-Subbarow (1925) after incubation 1 hr at 55°C and pH 8.0 with or without 1mM calcium. No significant change in phosphorus occurred with addition of calcium.

Effect of tannase

Tannase decomposes phenolic compounds with at least two phenolic hydroxyl groups. Optimal conditions are pH 6.0 and 35°C. Similar conditions were chosen in soaking trials with addition of tannase (Table 5). Best results were achieved by incubation of flour 1 for 4 hr in 37°C de-ionized water (pH 6.4). The remaining phytate was 49% lower when tannase was added ($p < 0.01$) than without tannase. No effect of addition of tannase was found when incubating in citrate-buffer at pH 6.0. Incubating flour 2 in water or citrate-buffer at similar conditions was not affected by tannase. In one set of experiments flour 1 was first soaked in pH 6.0 for 2 hr and then in pH 8 for 2 hr. The aim was to first degrade polyphenols with tannase and then incubate the flour at optimal conditions to reduce phytate. This did not increase phytate degradation much, only 30% was de-

Table 7—Fermentation of whole brown beans pre-soaked for 17 hr in 37°C de-ionized water and dried in a hot air oven for 24 hr[a]

Time (hr)	Temp (°C)	IP_6[b] µmol/g	IP_5 µmol/g	IP_4 µmol/g	IP_3 µmol/g	% reduction of IP_6
24 hr	21	4.7 ± 0.34	0.5 ± 0.04	0.3 ± 0.02	0.2 ± 0.04	63
f.H_2O[c]		2.5 ± 0.15	0.3 ± 0	0.1 ± 0.01	n.d**	
24 hr+	21					
24 hr	37	1.6 ± 0.08	0.3 ± 0.01	0.1 ± 0.01	0.7 ± 0.19	87
f.H_2O[c]		n.d**	n.d**	0.1 ± 0	5.7 ± 0.07	
24hr+	21					
48 hr	37	1.8 ± 0.11	0.2 ± 0.01	0.1 ± 0.06	0.7 ± 0.37	86
f.H_2O[c]		n.d**	n.d**	n.d**	1.3 ± 0.09	

[a] Values are µmol/g freeze-dried sample and are given as means ± S.D. for duplicate samples.
[b] IP_6-IP_3: Inositol hexa-, penta-, tetra- and triphosphate.
[c] f.H_2O: Fermentation wate which was decanted before freeze-drying of the whole, fermented beans.
** n.d: not detectable

graded compared to 53% degradation when tannase was not added.

Effect of fermentation

Fermentation of flour from beans that had been pre-soaked (flour 2) resulted in a reduction of phytate by 68% after 48 hr (table 6). Fermentation of whole pre-soaked beans was more favorable for phytate reduction than fermentation of ground beans ($p < 0.01$). Phytate was degraded by 86% after 48 hr and the fermentation water had a high content of inositol triphosphate. Fermentation for 72 hr did not increase phytate reduction (Table 7).

DISCUSSION

Optimal conditions for phytase

Phytase of brown beans (*Phaseolus vulgaris* L.) had optimal activity at pH 7 and 55°C which differed from the acid optima for phytase activity reported for cereals. The phytase also had a high activity at pH 8 and 37°C in agreement with Scott (1991) who demonstrated that bean phytases had higher activities at pH 8 and 37°C than at pH 5 and 37°C. In other studies pH optima of bean phytases were 5.2–5.3 (Becker et al., 1974; Chang et al., 1977; Gibbins and Norris, 1963; Gibson and Ullah, 1988; Lolas and Markakis, 1977; Sloane-Stanley, 1961). However, only one of those studies included neutral pH (Becker et al., 1974).

Inositol tetra- and triphosphate did not degrade within 17 hr when soaking at pH 7 and 55°C but accumulated. Thus the bean phytase active at pH 7 may be specific for inositol hexa- and pentaphosphates as substrates or may have been inhibited by inositol tetra- and triphosphates. The small increase in free myo-inositol when the bean flour was incubated at pH 7.0 also suggested incomplete degradation of inositol phosphates. The content of free myo-inositol increased more when the flour was incubated at pH 4.5 and there was no accumulation of inositol tetra- and triphosphate. This indicated that some phytase or phosphatase had acid activity and could use inositol tetra-, tri-, di- and monophosphates as substrates.

The sum of inositol hexa- and pentaphosphates after incubation 1 hr in tris-buffer pH 7.0 at 55°C was very low (0.45 µmol/g) while content of inositol tetra- and triphosphates was still high (2.33 µmol/g and 10.37 µmol/g, respectively). The contents of inositol hexa- and pentaphosphates were low enough that they would not have a strong negative effect on bioavailability of iron (Sandberg and Svanberg, 1991). The effect of inositol tetraphosphate and inositol triphosphates on iron absorption is not clear. Previous studies in vitro (Sandberg et al., 1989), in animals (Lönnerdahl et al., 1989) and in humans (Sandström and Sandberg, 1992; Sandberg et al., 1993) indicated that inositol hexa- and pentaphosphates, added separately in a white wheat roll formulation, reduced iron and zinc bioavailability. Tetra- and triphosphates of inositol had no such effect. Thus, probably the remaining inositol tetra- and triphosphates would not affect mineral absorption. Remaining iron binding phenolic compounds that could affect bioavailability of iron was not determined.

Addition of calcium

The alkaline phytase in brown beans (*Phaseolus vulgaris* L.) was not activated by 1 mM $CaCl_2$ ($p > 0.05$). Studies on other beans (Scott, 1991; Gibson and Ullah, 1988) have shown that purified bean phytases were activated by 1 mM $CaCl_2$. The difference with our results was that we studied whether an activation would occur when 1 mM $CaCl_2$ was added to ground beans rather than to the purified enzyme. The beans contain 135 mg calcium/g dried beans, and thus the total concentration of calcium in our trial was 338 mM.

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Texture and Pectic Composition Differences in Raw, Cooked and Frozen-Thawed Chinese Cabbages due to Leaf Position

MICHIKO FUCHIGAMI, NORIKO HYAKUMOTO, and KOICHI MIYAZAKI

ABSTRACT

Differences in texture of raw, cooked and blanched-frozen-then-thawed Chinese Cabbages and their leaf positions (10, 20, 30, 40, or 50th midribs from the outside) were investigated. Firmness and rupture strain of raw midribs were low, but increased after short cooking time. Crispness of tissues decreased and elasticity increased. The 20 ~30th midribs were slowest to soften during cooking, while the inner midribs from the 40th were more readily softened. The 40th contained more high methoxyl pectin than did the 20th. Therefore, they were more quickly softened because of pectic release by transelimination. After freezing-thawing, firmness decreased and rupture strain increased. Midribs with firmer texture contained more pectin. After freezing-thawing, amounts of pectin in midribs decreased.

Key Words: Chinese cabbage, texture, pectin, freezing, leaf position

INTRODUCTION

LEAFY VEGETABLES are often eaten with frozen noodles. However, the texture of frozen leafy vegetables, especially Chinese cabbage, is undesirable (mesophyll too soft and fibers firm). Improvement in textural quality of frozen leafy vegetables is important, but difficult for frozen Chinese cabbage. Since chinese cabbage contains high moisture and thin cell walls, freezing effects are notable. Basic research has indicated it was difficult to measure texture of vegetables with leafy heads. Size, weight and thickness of midribs of Chinese cabbage varied in life stage (Saito, 1983) or in position of leaves (outer or inner; top, middle or bottom). Therefore, consistent sample preparation was very unreliable. With leaves positioned more inward, the amount of sugars increased and freezing points decreased (30th from outside: midrib −1.5°C, leaf −2.2°C) (Yamane, 1982). However, measured results on texture of raw, cooked and frozen Chinese cabbage as related to leaf position have not been reported.

Our objective was to measure differences in position of leaves and their relation to the firmness of tissues and pectic composition in raw, cooked or frozen-thawed Chinese cabbage midribs.

MATERIALS & METHODS

Sample preparation

Chinese cabbage (*Brassica pekinensis* Rupr., Ohsho) with fresh heads harvested in January in Okayama, Japan, were used; sample A, 2.5 kg; sample B, 4 kg. The midribs of leaves numbered 10, 20, 30, 40 or 50 (from outside to inside) were cut into three segments (top, middle and bottom) and then cut into 2 cm × 2 cm pieces. These were designated as the 10th~50th midribs (Fig. 1).

Cooking, freezing and thawing

The four pieces of samples were blanched 1 min in boiling water. Blanched samples were cooked 1 min or frozen at −30°C using a conventional freezer, and then thawed 40 min at 20°C or cooked 1 min in boiling water. Experiments were repeated two times.

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Measurement of texture

The firmness of raw, cooked, frozen-thawed sample A was measured using a rheometer (NRM-2002J, Fudokogyo Co., Tokyo) with plunger (interpenetrated type). The firmness and rupture strain of sample B were measured using a creepmeter (RE-33005, Yamaden Co., Tokyo) as reported previously (Fuchigami et al., in press). The thickness of samples was measured using a sample-height counter (HC-3305, Yamaden.Co., Tokyo), and then samples were compressed to 0.01mm. Maximum force (breaking load) and the distance of breaking point ÷ the thickness of sample × 100 were used as indexes of firmness (N /mm^2) and rupture strain (%), respectively. In both measurements, plungers of cylindrical shape (3 mm in diameter) were used avoiding veins.

Extraction and determination of pectic substances

Method 1. Pectic substances in the 20th and 40th midribs were extracted successively with four reagents: 0.01N HCl (pH 2.0, at 35°C, 24 hr×5), 0.1M sodium acetate buffer (pH 4.0, at 35°C, 24 hr×3), 2% sodium hexametaphosphate (pH 4.0, at 90°C, 3.5 hr×3), and 0.05N HCl (at 90°C, 4 hr×3) solutions. These fractions were designated as PA, PB, PC, and PD, respectively. Method I was effective for fractional extraction of pectic substances with different degrees of esterification (Fuchigami and Okamoto 1984; Fuchigami, 1987). However, extraction was time-consuming and therefore method 2 (Bettelheim and Sterling, 1955; Miura and Mizuta, 1962) was also used for total pectic substances. This method effectively determined water soluble pectin (WSP) and total pectin, but was not suitable for fractionation of low and high methoxyl pectins (Fuchigami and Okamoto, 1984).

Method 2. Alcohol insoluble solids (AIS) were prepared from the 10th, 30th and 50th midribs. Pectic substances were extracted from AIS successively with three reagents; distilled water (at 20°C, 24 hr), 0.4% sodium hexametaphosphate (at 20°C, 24 hr) and 0.05N HCl (at 85°C, 4 hr). The reagents were exchanged one time during extraction. These fractions were designated as WSP, PSP and HSP, respectively. The amount of galacturonic acid was determined by the carbazole method (Galambos, 1969).

pH value and soluble sugar in raw midribs

The middle segments of midribs were added to an equal weight of distilled water then homogenized and filtered. The pH values of supernatants were measured. The amount of soluble sugar in supernatants was determined using a digital sugar meter (Pallet 100 PR-100, Atago Co., Tokyo) and was indicated ×2. Experiments were repeated three times.

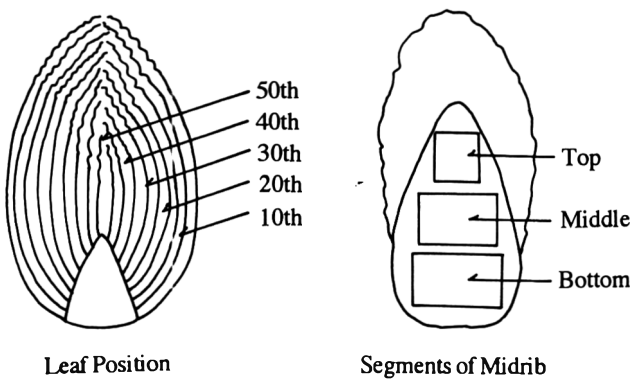


Fig. 1—Leaf position and segments of midrib.

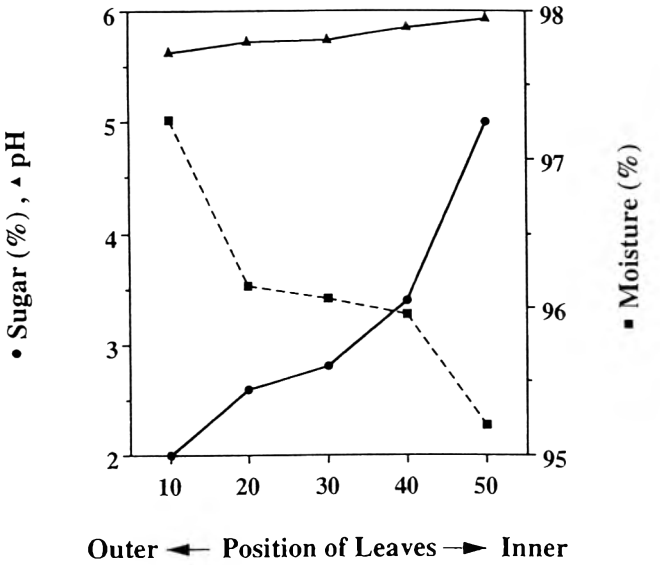


Fig. 2—pH, moisture, and soluble sugars in midribs as related to leaf position.

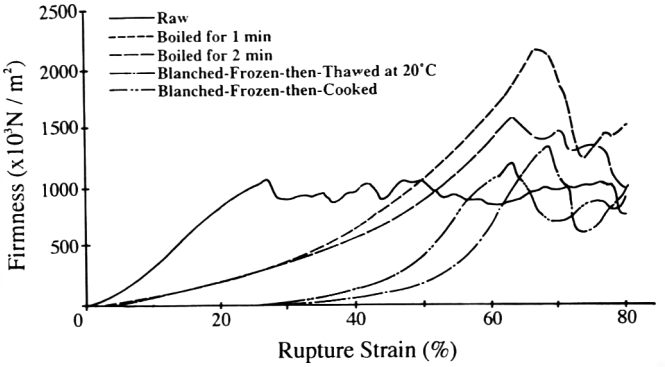


Fig. 3—Force-distance curves of raw, cooked and blanched-frozen-thawed midribs by creepmeter.

RESULTS & DISCUSSION

pH, moisture and soluble sugar in midribs

Differences in pH, moisture and soluble sugar among midribs of different positions were compared (Fig. 2). Moisture in the 50th midrib was least (95.2%) but increased as leaf position moved outward; the 10th was greatest (97.3%). Conversely, soluble sugar in the 50th was greatest (5%) while that of the 10th was least (2%). Sensory evaluation after 2 min cooking indicated the 1~10th midribs were not so sweet, but inner midribs from the 20th were sweet. The outer leaves photosynthesized actively, and photosynthates were stored in the inner leaves. The pH value (diluted two times) of the 50th was highest (5.95) and that of the 10th was lowest (5.65).

Firmness of top, middle and bottom segments of raw, cooked and frozen-thawed midribs

Rheometer measurements of firmness of raw, cooked and blanched-frozen-thawed midribs (sample A) were compared (Table 1). No difference in firmness of top, middle and bottom segments of raw midribs was observed. The top segments became firmer after 1 min cooking and then became softer than raw midribs after 2 min cooking. However, the 10th and 20th of the middle segments and all bottom segments became firmer than raw midribs even after 2 min cooking, with bottom segments apparently the most difficult to soften. The thickness of midribs increased as position was closer to the bottom, so these segments were more difficult to soften during short-time cooking.

The greater firmness reading of 1 min cooked midribs (compared to raw) might be due to the measuring device compressing against the epidermis. When the epidermal tissues were peeled off, 1 min cooked mesophyll softened more than raw. The short time cooked epidermis might have been difficult to break using a plunger (3 mm i.d.), because of its elasticity.

Blanching-freezing then thawing at 20°C or 100°C decreased firmness of midribs compared those cooked 1 min or 2 min ($p<0.001$). The 20~30th midribs were the most difficult to soften during cooking, but the 40th was easily softened. Sensory evaluation after freezing-then-cooking indicated that the 10th epidermis was firm, but both epidermis and mesophyll increased in softness as position changed inward from the 20th. The firmness of cooked or thawed segments was least to greatest: top < middle < bottom ($p<0.001$).

Firmness and rupture strain of raw, cooked and frozen-thawed midribs (10th~50th)

The typical force-distance curves of raw, cooked and blanched-frozen-thawed middle segments (30th) of sample B, measured using a creepmeter, were compared (Fig. 3). These curves showed changes in textural properties from the point of foods touching teeth to the meeting of upper and lower teeth. When samples were compressed to 10% of their thickness, the raw midrib was six times firmer than the other samples. However, after 20% compression the greatest difference in firmness among the three groups (raw, cooked, frozen-thawed) became apparent. The slope of the curve for the raw midrib was greater than other curves. Therefore, raw samples were firmer than other groups, but they broke when they were compressed 25%. Final firmness of raw samples was less than cooked samples. Both firmness and rupture strain of raw midribs were small, but about

Table 1—Difference in the firmness of top, middle and bottom segments of raw, cooked and frozen-then-thawed micribs by rheometer ($\times 10^3$ N /m^2)

Segments of midribs	Leaf position	Raw (R)	Boiled 1 min (B1)	Boiled 2 min (B2)	Blanched-frozen then-thawed (FT)	Blanched-frozen then-cooked (FC)	T-test	R	B1	B2	FT	FC
	10th	777± 32	961± 76	734± 53	684±36	411± 1		–	–	–	–	***
Top	20th	891± 47	974± 72	868± 64	781±24	444± 17	T:M	–	***	**	***	***
(T)	30th	925± 49	1043± 58	763± 43	859±24	480± 79		–	–	**	–	**
	40th	828± 36	857± 92	565± 74	634±61	272± 44		–	*	***	*	*
	10th	793± 47	1056±124	843±118	709± 71	580± 12		*	–	**	–	–
Middle	20th	892± 40	1296± 69	1068±136	846± 6	657± 50	M:B	–	–	*	***	**
(M)	30th	906± 54	1136± 93	872± 58	845±79	638± 50		–	***	***	–	–
	40th	924± 54	1029±136	848± 65	720±55	336± 20		**	**	***	–	–
	10th	881± 54	1146± 76	1058± 98	780±49	627± 94		**	***	***	**	***
Bottom	20th	997±104	1316± 58	1243± 98	975±71	779± 97	T:B	–	***	***	***	***
(B)	30th	982± 55	1416± 85	1187±111	745±62	644±122		–	***	***	–	–
	40th	1018± 24	1289±103	1078± 93	752±46	505±132		**	**	***	–	*

– $p>0.05$, * $p<0.05$, ** $p<0.01$, *** $p<0.001$: significant differences between segments (top: middle: bottom).

Table 2—Firmness of raw, cooked and frozen-then-thawed midribs (middle segments) by creepmeter ($\times 10^3$N /m^2)

Leaf position	Raw (R)	Boiled 1 min (B1)	Boiled 2 min (B2)	Blanched-frozen then-thawed (FT)	Blanched-frozen then-cooked (FC)	T-test R:B1	T-test B1:B2	T-test B1:FT	T-test B2:FC
10th	989 ± 164	1632 ± 168	1369 ± 250	1212 ± 132	1251 ± 90	***	–	***	–
20th	1233 ± 269^{-a}	1985 ± 51^{***a}	1552 ± 49^{-a}	1201 ± 122^{-a}	1193 ± 32^{-a}	***	***	***	***
30th	1121 ± 119^{-b}	2019 ± 370^{-b}	1418 ± 125^{*b}	1272 ± 121^{-b}	1165 ± 140^{-b}	***	**	***	***
40th	1114 ± 71^{-c}	1574 ± 169^{*c}	1015 ± 96^{***c}	600 ± 69^{***c}	619 ± 100^{***c}	***	***	***	***
50th	1054 ± 129^{-d}	1126 ± 83^{***d}	677 ± 50^{***d}	581 ± 74^{-d}	429 ± 104^{*d}	–	***	***	***

– $p>0.05$, * $p<0.05$, *** $p<0.001$: significant differences between leaf positions (^{a} 10th : 20th, ^{b} 20th : 30th, ^{c} 30th: 40th, ^{d} 40th : 50th) by T-test.

Table 3—Rupture strain of raw, cooked and frozen-then-thawed midribs (middle segments) by creepmeter (%)

Leaf position	Raw (R)	Boiled 1 min (B1)	Boiled 2 min (B2)	Blanched-frozen then-thawed (FT)	Blanched-frozen then-cooked (FC)	T-test R:B1	T-test B1:B2	T-test B1:FT	T-test B2:FC
10th	26.1 ± 10.2	55.8 ± 2.8	60.1 ± 1.3	65.9 ± 2.1	64.8 ± 3.0	***	**	***	**
20th	30.7 ± 6.9^{-a}	66.7 ± 1.5^{***a}	63.1 ± 1.5^{**a}	71.4 ± 3.5^{**a}	64.8 ± 2.8^{-a}	***	**	*	–
30th	28.6 ± 1.9^{-b}	65.5 ± 4.2^{-b}	62.6 ± 2.7^{**b}	66.3 ± 3.1^{*b}	64.5 ± 4.3^{-b}	***	–	–	–
40th	26.4 ± 2.4^{-c}	63.9 ± 1.7^{-c}	57.1 ± 1.7^{***c}	60.4 ± 7.2^{-c}	63.0 ± 3.0^{-c}	***	***	*	***
50th	25.6 ± 5.0^{-d}	60.0 ± 3.1^{*d}	53.3 ± 2.7^{-d}	57.3 ± 3.1^{-d}	57.1 ± 5.6^{-d}	***	**	–	–

a,b,c,d: see Table 2.

Table 4—Thickness (mm) of raw, cooked and frozen-then-thawed midribs (middle segments) by creepmeter

Leaf position	Raw (R)	Boiled 1 min (B1)	Boiled 2 min (B2)	Blanched-frozen then-thawed (FT)	Blanched-frozen then-cooked (FC)	T-test R:B1	T-test B1:B2	T-test B1:FT	T-test B2:FC
10th	7.86 ± 0.52	6.59 ± 0.27	6.51 ± 0.44	3.63 ± 0.26	3.76 ± 0.21	***	**	***	***
20th	8.07 ± 0.78^{-a}	7.26 ± 0.34^{**a}	6.95 ± 0.77^{-a}	5.47 ± 0.66^{***a}	4.49 ± 0.12^{***a}	–	–	***	***
30th	9.02 ± 0.51^{*b}	7.69 ± 0.43^{-b}	7.36 ± 0.67^{-b}	5.67 ± 0.40^{-b}	5.14 ± 0.49^{*b}	***	–	***	***
40th	7.88 ± 0.45^{**c}	7.09 ± 0.35^{*c}	6.50 ± 0.41^{*c}	4.57 ± 0.36^{***c}	4.64 ± 0.23^{-c}	**	–	***	***
50th	7.47 ± 0.87^{-d}	5.93 ± 0.21^{***d}	6.23 ± 0.70^{-d}	4.74 ± 0.20^{-d}	4.51 ± 0.54^{-d}	***	–	***	***

a,b,c,d: see Table 2.

Table 5—Differences in pectic composition between 20th and 40th leaves of raw, cooked and frozen-then-thawed midribs

Pectic composition[a]	Raw		Boiled 1 min		Boiled 2 min		Blanched-frozen then-thawed		Blanched-frozen then-cooked	
Leaf position	20th	40th	20th	40th	20th	40th	20th	40th	20th	40th
PA	118	186	97	169	126	183	124	147	113	102
PB	216	159	215	161	180	140	169	113	175	143
PC	103	84	105	77	78	61	74	78	90	95
PD	41	22	38	23	34	23	29	29	35	35
Total	478	451	455	430	418	407	396	367	413	375

The amount of galacturonic acid was determined on a fresh weight basis (mg /100g).
[a] PA: 0.01N HCl soluble pectin; PB: 0.1M acetate buffer soluble pectin; PC: 2% sodium hexametaphosphate soluble pectin; PD: 0.05N HCl soluble pectin.

the same firmness was maintained during 25%~80% compression. Therefore raw midribs were described as "crisp."

When the samples were cooked for 1 or 2 min, angles of initial inclination of curves decreased. Therefore, cooked samples compressed to 50% were softer than raw samples, but above 50% compression not only firmness but also rupture strain increased. Short-time cooking (e.g. blanching) increased the elasticity of epidermis and softening of mesophyll. The texture of cooked midribs differed greatly from raw midribs.

After blanching-freezing then thawing, firmness and rupture energy of midribs decreased. Firmness of 30% compressed midribs was 0, and then curves were concave downward. This shape is typical of products such as marshmallows and softer fruits and vegetables (Bourne, 1982). On the other hand, the initial curve of raw midrib was linear; this curve is found with rigid products such as firm green fruits and vegetables (Bourne, 1982). Evidently texture of midribs changed considerably during freezing-thawing.

The averages of four measurements for firmness, rupture strain, and thickness of 10th–50th midribs of sample B after cooking and freezing, then thawing, were compared (Tables 2–4). Sample B (Table 2) was firmer than sample A (Table 1) in all treatments, either because sample B was larger than A or because two different measuring devices were used. After cooking or freezing-thawing, firmness of the 20–30th midribs was greatest while inner midribs from the 40th softened readily ($p<0.001$).

The rupture strain of raw samples was 25–30%, but of cooked or frozen-thawed samples was 55–70% ($p<0.001$) (Table 3). The rupture strain of the 20th midrib was greatest, and as position changed inward it decreased. The thickness of the 30th was greatest (Table 4). Thickness of midribs was greatest to least; raw > cooked for 1 min > cooked for 2 min > frozen-thawed at 20°C > frozen-thawed at 100°C. Cooking, freezing and thawing decreased thickness of the samples. Chinese cabbage was very high in moisture (95–97.5%); therefore, loss of water from tissues due to drip during thawing at 20°C (10–15%) or at 100°C (30–35%) changed the texture.

Pectic composition of raw, cooked and frozen-thawed midribs (10th~50th)

Changes in pectic composition of 20th and 40th midribs during cooking or freezing-thawing (by method 1) were compared (Table 5). The 20th midrib contained more pectic substances than the 40th. However, the raw 40th midrib contained more PA (high methoxyl pectin) and less PB, PC and PD (low methoxyl pectins) than did the 20th midrib. The amount of pectic substances in the 40th midrib after cooking was less than that in the 20th. The results suggest that the inner midribs from the 40th contained more PA which was easily broken down through transelimination and had less PB, PC and PD which were difficult to degrade by cooking; therefore, they were easily softened

Table 6—Differences in pectic composition between 10th, 30th and 50th leaves of raw, cooked and frozen-then-thawed midribs

Pectic composition[a]	Raw			Boiled 1 min			Boiled 2 min			Blanched-frozen then-thawed			Blanched-frozen then-cooked		
Leaf position	10th	30th	50th	10th	30th	50th	10th	30th	50th	10th	30th	50th	10th	30th	50th
WSP	6	4	10	12	22	23	9	15	28	10	12	11	10	11	19
PSP	136	161	122	153	182	148	143	195	132	141	164	127	157	176	131
HSP	306	286	313	202	224	251	211	218	226	206	242	225	189	222	210
Total	448	451	445	367	428	422	363	428	386	357	418	363	356	409	360

The amount of galacturonic acid was determined on a fresh weight basis (mg /100g).
[a] WSP: water soluble pectin; PSP: 0.4% sodium hexametaphosphate soluble pectin; HSP: 0.05N HCl soluble pectin.

by cooking. More PA might be easily extracted by depolymerization with 2 min boiling compared to 1 min boiling.

Through freezing-then-thawing, the amount of pectin in the 40th midribs decreased more than that of the 20th, but no difference occurred between thawing methods. Plants usually resist freezing in winter by increased soluble sugar content (Sakai, 1982). The amount of soluble sugar in Chinese cabbage was greater as position changed toward the inside. However, the firmness of frozen-thawed inner midribs was softer and loss of pectin by freezing-thawing was greater than in the 20th~30th midribs. Results suggested that the effect of pectic loss by blanching and cooking on the softening of tissues was greater than the effect of sugar on freezing tolerance. Texture of the frozen raw midribs might be affected by the amount of sugars due to decrease of freezing point (Yamane, 1982).

Changes in pectic composition of 10th, 30th and 50th midribs during cooking or freezing-thawing (by method 2) were also compared (Table 6). Little WSP was found in raw midribs, and WSP remaining in midribs increased slightly by cooking or freezing-thawing; 50th > 30th > 10th. Total pectin remaining in midribs after cooking or freezing-thawing was greatest to least; the 20th or 30th > 40th > 50th > 10th. The reason for decrease of pectin in the 10th by cooking was not clear without determination by method 1. However, physiological properties of outer leaves (the 10th) differ from other leaves (inner from the 20th) (Saito, 1983). Results suggested that pectin was more readily decomposed in the inner midribs. Compared to changes in firmness, the midribs which had a firmer texture contained more pectin.

Thus, pectic composition of Chinese cabbages varied with leaf positions and affected the softening of midribs after cooking or blanching-freezing-thawing.

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1-Cyano-2,3-Epithiopropane as the Primary Sinigrin Hydrolysis Product of Fresh Cabbage

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ABSTRACT

Dichloromethane extracts of juices from fresh cabbages, including four known (Brutus, Galaxy, Bentley, Structon) and two unknown cultivars, were analyzed by GC-MS for the presence of sinigrin degradation products. Allyl isothiocyanate (AITC), which has been reported to be the important aroma compound of freshly disrupted cabbage was not detected in any of the dichloromethane extracts of cabbage juice. Instead, 1-cyano-2,3-epithiopropane, which is one of the isomers of AITC, was the primary volatile compound in all cabbage extracts. AITC was detected in relative trace concentration only when cabbage juice was injected into the GC, using a wide bore, packed column. Thus, the relative importance of AITC and 1-cyano-2,3-epithiopropane to the aroma of freshly disrupted cabbage should be considered.

Key Words: 1-cyano-2,3-epithiopropane, sinigrin, allyl isothiocyanate, cabbage

INTRODUCTION

HEWITT ET AL. (1956) witnessed the generation of cabbage flavor when they added an enzyme preparation from cabbage and mustard seeds into rehydrated dehydrated cabbage. The first report we found of the presence of allyl isothiocyanate (AITC) in cabbage, however, was made by Mackay and Hewitt (1959). They reported AITC was generated when they repeated the investigation of Hewitt et al. (1956) by adding a thioglucosidase (thioglucoside glucohydrolase, myrosinase) preparation from cabbage and mustard seeds into rehydrated dehydrated cabbage. AITC also has been reported by other scientists to be one of the important aroma compounds of fresh (Clapp et al., 1959; Bailey et al., 1961; Chin and Lindsay, 1993; Schwimmer, 1963) and heated (MacLeod and MacLeod, 1970) cabbage, along with other volatile sulfur compounds. The concentration of allylglucosinolate (sinigrin), the precursor of AITC, was reported to be 17-612 ppm (Van Etten et al., 1976). Four different volatile compounds (AITC, allyl cyanate, allyl thiocyanate and 1-cyano-2,3-epithiopropane) have been reported to be generated from myrosinase hydrolysis of sinigrin, depending on reaction conditions (Springett and Adams, 1988).

However, during the course of our investigations on the antibacterial activity of fresh cabbage juice (Kyung and Fleming, 1994a, b), we did not detect AITC. Instead, we tentatively identified a prominent GC peak as 1-cyano-2,3-epithiopropane based on GC retention time and MS characteristics (Kyung and Fleming, 1994b).

1-Cyano-2,3-epithiopropane has been reported to occur in disrupted, fresh cruciferous vegetables (Cole, 1975; 1976; Daxenbichler et al., 1977; Petroski and Tookey, 1982) and in aqueous suspensions of crambe seed flour (Luthy and Benn, 1979) or cabbage seed meal (Kaoulla et al., 1980), but not in white mustard seed or horseradish (Petroski and Tookey, 1982). Both AITC and 1-cyano-2,3-epithiopropane are generated from a common precursor, allylglucosinolate (sinigrin, Fig. 1), by the action of thioglucosidase. Cole (1975) indicated that different thioglucosidases produced different end products from the same substrate. Tookey (1973), however, isolated a protein in the seeds of *Crambe abyssinica*, which was required for the formation of cyanoepithioalkane. He termed it epithiospecifier protein (ESP). Following the discovery of ESP in crambe seeds, Petroski and Tookey (1982) isolated ESP from various cruciferous vegetables. Thioglucosidase enzyme alone produced only alkenyl isothiocyanates from alkenylglucosinolates, while the enzyme along with ESP generated the corresponding cyanoepithioalkane. White mustard seed and horseradish generated only isothiocyanate because they lacked ESP (Petroski and Tookey, 1982). Thioglucosidase from those plants, however, generated 1-cyano-2,3-epithiopropane from sinigrin when ESP obtained from cruciferous vegetables was added.

Our objective was to verify our hypothesis that 1-cyano-2,3-epithiopropane, and not AITC, is the primary sinigrin hydrolysis product in homogenized fresh cabbage.

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MATERIALS & METHODS

CABBAGE CULTIVARS (cvs.) Brutus, Galaxy, Bentley and Structon were obtained from Castle Harvester Company, Inc. (Seneca Castle, NY). Two unknown cultivars of cabbage and one of horseradish were purchased from local stores. AITC, thioglucosidase, and dichloromethane were purchased from Sigma Company (St. Louis, MO).

Preparation of juices from cabbage and horseradish

Juices from vegetables were extracted by an electrical centrifuge-type juice extractor (Braun, Germany) as described by Kyung and Fleming (1994a). Cabbage juice augmented with AITC (50 ppm) was made by adding AITC into a portion (50 mL) of cabbage juice.

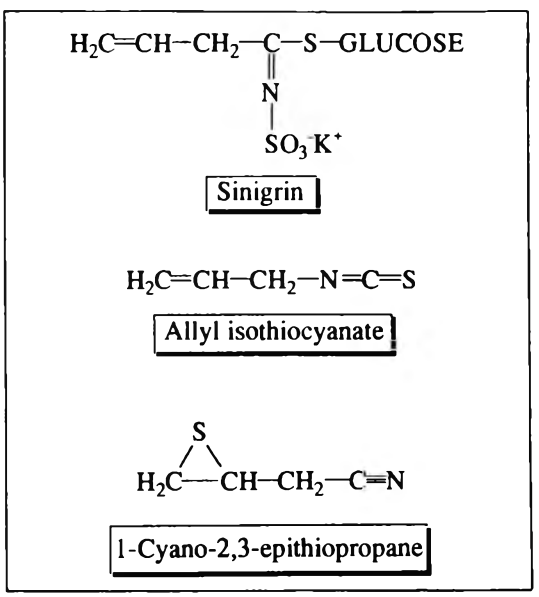


Fig. 1—Sinigrin and two of its hydrolysis products.

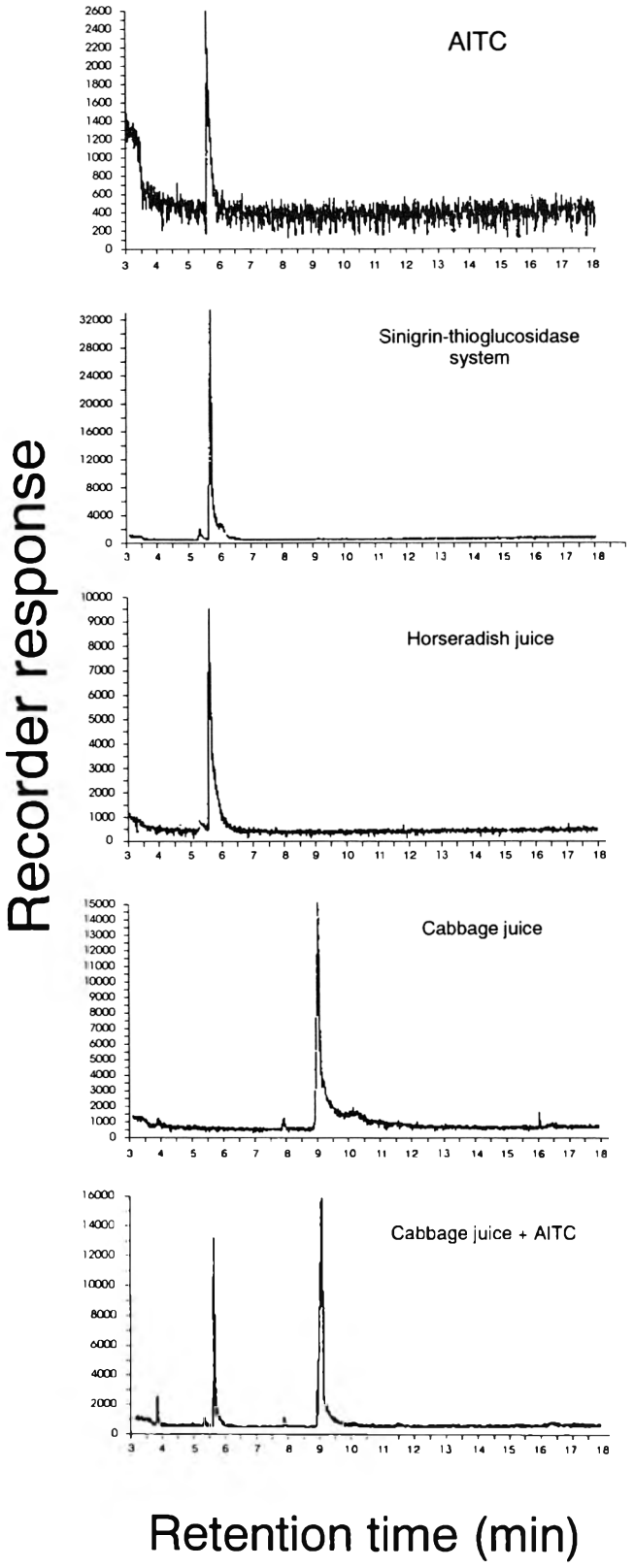


Fig. 2—Gas chromatograms of dichloromethane extracts of aqueous solutions. The analyses in Fig. 1 and 2 were obtained by combination GC/MS. A DB-5-coated, capillary column was used.

Preparation of an aqueous solution of AITC and sinigrin-thioglucosidase system

AITC standard was made by adding AITC into 50 mL of distilled water to make a final concentration of 50 ppm. Sinigrin (5 mg) and thioglucosidase (0.5 mg) were dissolved in 5 mL of 0.2M phosphate buffer (pH 6.2) and the mixture was kept at room temperature (≈23°C) for 30 min before extraction.

Dichloromethane extraction of volatiles

Aliquots of the preparations (50 mL each of aqueous solutions of AITC, cabbage juice, cabbage juice augmented with AITC and 5 mL each of sinigrin-thioglucosidase system and horseradish juice) were extracted with 5 mL of dichloromethane. The extracts were either analyzed immediately or stored at −83°C until analysis.

Identification of volatile compounds

Mass spectra of the isolated compounds were obtained by combination GC/MS (Model HP 5985B mass spectrometer and RTE VI data system, Hewlett-Packard, Palo Alto, CA). Volatiles in dichloromethane extracts were qualitatively analyzed. AITC and 1-cyano-2,3-epithiopropane were the compounds of primary interest. Portions of dichloromethane extract (2 or 3 µL) were injected for GC/MS analysis. The GC column (30 m capillary, J & W Scientific, Inc., Folsom, CA) was coated with DB-5 (0.25 µm thickness), and the oven temperature was programmed from 40 to 200°C at 5°C/min, with the initial temperature held 1 min. The carrier gas was He (1.5 mL/min). Samples were injected in the spitless mode. The injector port and detector were both at 150°C. Electron impact ionization, with an ionization potential of 70eV, was used and the mass range scanned was 40–200 daltons.

The volatile sulfur compounds were also analyzed under different conditions using a Shimadzu GC (Columbia, MD) with a column packed with 80–100 mesh Porapack P (Waters, Milford, MA). Two µL each of dichloromethane extract of cabbage juice, and of cabbage juice were directly injected. For headspace analysis, 1g of cabbage juice was weighed into 10 mL headspace glass vials (Tekmar Co., Cincinnati, OH). Juice samples were heated in a Tekmar 7000 Headspace Autosampler for 5 min at 100°C. The vials were pressurized to 103 kPa for 0.2 min and equilibrated for 0.2 min. The headspace was injected onto a 2 mL sampler loop for 0.5 min, equilibrated for 0.2 min, and the vapors from the loop were injected into the sampler line for 1 min. The sampler loop and line were held at 50°C, and vapors were collected with 34 kPa back pressure. The GC column was programmed from 105 to 225°C at 15°C/min, with initial temperature held for 0.5 min. A flame photometric detector was used. The carrier gas was N_2 (55 mL/min).

RESULTS & DISCUSSION

DICHLOROMETHANE EXTRACTS of cabbage showed one major peak (RT = 9.0 min), which had a longer retention time (RT) than authentic AITC (RT = 5.7 min; Fig. 2). No peak matching the RT of AITC appeared when dichloromethane extract was concentrated ≈50 times (data not shown). Authentic AITC added into cabbage juice showed the same RT as the AITC standard (Fig. 2). Extracts of both the sinigrin-thioglucosidase system and horseradish gave RT values identical to AITC (Fig. 2), confirming that AITC was produced as expected. There are reports that 1-cyano-2,3-epithiopropane has a longer RT than AITC (Cole, 1975; Daxenbichler et al., 1977; Kaoulla et al., 1980). The molecular ion at m/z 99 was the base peak, and the following losses are proposed to explain the observed fragment ions: loss of HCN (to give m/z 72), loss of $\cdot CH_2CN$ (to give m/z 59), and loss of $\cdot CH{=}S$ (to give m/z 54). These losses are consistent with the 1-cyano-2,3-epithiopropane structure. This structure also compared very well with published mass spectral data of 1-cyano-2,3-epithiopropane (Cole, 1975; Luthy and Benn, 1979; Kaoulla et al., 1980; Spencer and Daxenbichler, 1980; Springett and Adams, 1988). The latter compound has been reported in cruciferous vegetables (Cole, 1975; Daxenbichler et al., 1977; Luthy and Benn, 1979; Kaoulla et al., 1980; Spencer and Daxenbichler, 1980; Springett and Adams, 1988). When fresh cabbage was autolyzed, isothiocyanate peaks were essentially absent and nitriles, including 1-cyano-2,3-epithiopropane, were dominant (Daxenbichler et al., 1977). AITC, reported to be an important flavoring compound of fresh cabbage (Schwimmer, 1963; Chin and Lindsay, 1993), was not detected in our investigation. Also we did not detect AITC in our pre-

vious work (Kyung and Fleming, 1994b). Instead, we observed the presence of an unknown compound which had a mass spectrum matching 1-cyano-2,3-epithiopropane.

The dichloromethane extracts of the sinigrin-thioglucosidase system and horseradish were devoid of a 1-cyano-2,3-epithiopropane peak but showed a major peak with RT and mass spectrum matching authentic AITC (Fig. 2 and 3). AITC added into cabbage juice, as well as that in an aqueous solution, was detected by GC-MS and showed matching RT (Fig. 2) and mass spectrum (Fig. 3) with that of authentic AITC. The mass spectrum of AITC matched well with that of AITC in published reports (Bailey et al., 1961; Kaoulla et al., 1980). These evidences suggest that sample handling, including extraction and analysis of AITC from cabbage juice was not causing loss of the AITC, nor forming 1-cyano-2,3-epithiopropane through isomerization.

When dichloromethane extract of Brutus cabbage was analyzed under different conditions with a wide bore column packed with Porapack P, recorder response for 1-cyano-2,3-epithiopropane and AITC were 99.3 and 0.12, respectively (data not shown). When fresh cabbage juice was directly injected into GC, 1-cyano-2,3-epithiopropane was one of the major peaks, with AITC showing only a trace concentration (data not shown).

Based on the GC-MS characteristics of authentic AITC and the volatiles from cabbage, horseradish, and sinigrin-thioglucosidase system, the primary hydrolysis product of sinigrin in freshly disrupted cabbage appears to be 1-cyano-2,3-epithiopropane, and AITC is probably present only in relatively small concentrations, if any, in freshly disrupted cabbage.

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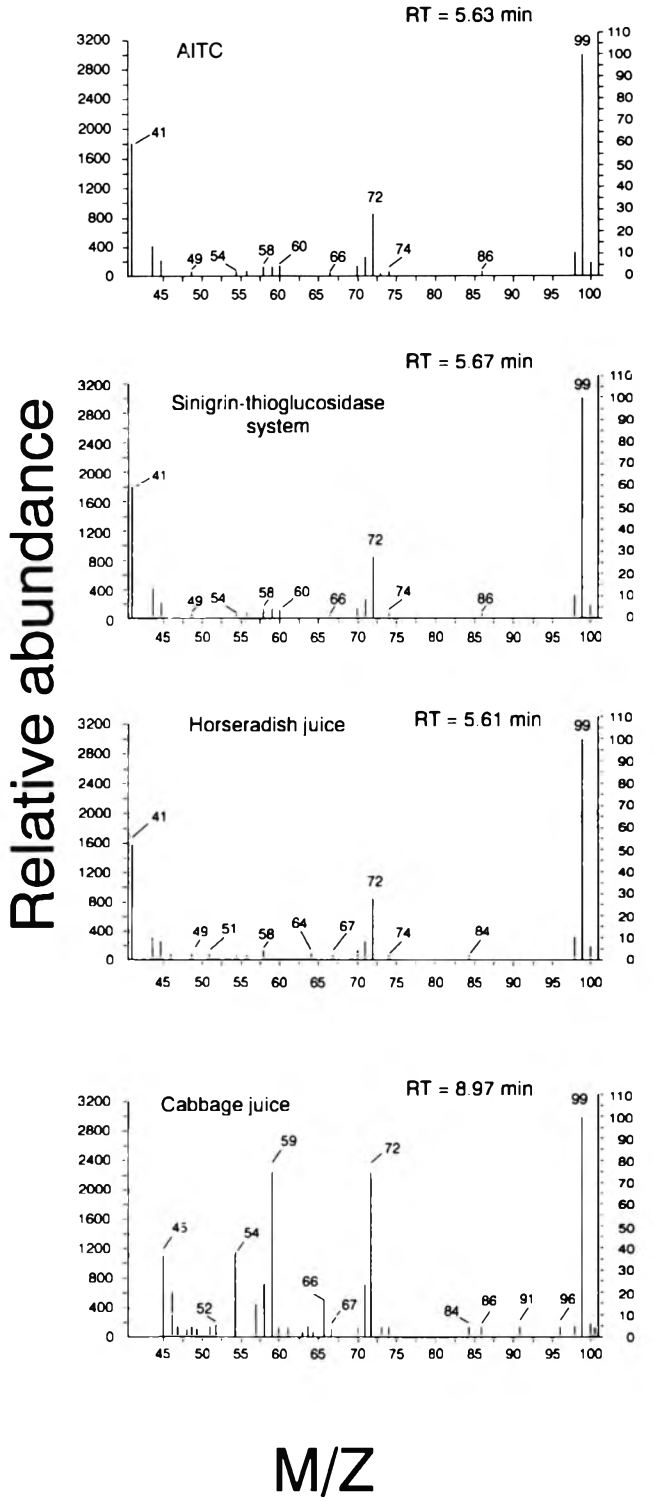


Fig. 3—Mass spectra of major compounds in dichloromethane extracts separated by GC.

Ms received 7/5/94; revised 8/26/94; accepted 9/20/94.

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Modification of Crude Canola Lecithin for Food Use

FERAL TEMELLI and NURHAN TURGUT DUNFORD

ABSTRACT

Crude canola lecithin was fractionated by removal of neutral lipids with acetone followed by ethanol extraction with or without supercritical carbon dioxide (SC-CO_2). Two extraction steps of 60 min each at 35°C with 500 mL acetone were optimum for maximum yield of acetone solubles and maximum phospholipid (PL) concentration (66.7%) in the acetone insoluble (AI) fraction. AI were separated into 26.5% ethanol soluble and 68.7% ethanol insoluble fractions. PL concentration in the extract increased with increasing amounts of ethanol used. Yields were low for SC-CO_2/ethanol extraction, of which extracts from 55.2 MPa/70°C contained 32% PL (83% phosphatidyl choline).

Key Words: canola lecithin, acetone extractables, supercritical CO_2, phospholipids

INTRODUCTION

"LECITHIN" commonly refers to a mixture of phospholipids dissolved in oil. Crude lecithin contains ≈60% phospholipids and 30% oil, dry basis. Lecithin is an important natural emulsifier used in foods, feed, pharmaceuticals and technical products. The major source of crude lecithin is the degumming step in soybean oil refining. Though, composition of canola lecithin (Vaisey-Genser and Eskin, 1987) resembles that of soybean, it is not utilized for food purposes and it is added back to meal which is used as animal feed.

The mixture of phospholipids in crude lecithin (phosphatidyl choline, PC; phosphatidyl ethanolamine, PE; phosphatidyl inositol, PI) gives weak water-in-oil (w/o) and oil-in-water (o/w) emulsifying properties. Improved emulsifying properties could be obtained by modification of crude lecithin. PC enriched lecithin has better o/w emulsifying properties than lecithin mixtures and it functions in wide pH ranges. PC has also therapeutic effects on various metabolic disorders such as lowering cholesterol levels, treating neurological disorders etc. (Hanin, 1979). Most of the research on modification of crude lecithin has been with soybean lecithin (Van Nieuwenhuyzen, 1976, 1981). Little has been published on improvement of crude canola lecithin properties. Industrial scale modification of crude soybean lecithin is achieved by physical, chemical and enzymatic methods. Oil removal from crude lecithin is usually achieved by solvent treatment. One such method involves treatment of crude lecithin with anhydrous acetone, where granulated phospholipid blends with 95–98% acetone insolubles (AI) have been obtained (Van Nieuwenhuyzen, 1981). Benzene-acetone mixtures can also be used for separation of oil from crude lecithin. Niewiadomski (1970) reported that acetone, as well as acetone-benzene refining, removed undesirable sensory properties of rapeseed lecithin, (such as a burnt odor), only to a limited extent.

Fractionation of crude lecithin into PC and PE rich portions can be achieved by alcohol treatment. By variables such as temperature, alcohol polarity, concentration, lecithin/alcohol ratio and treatment time, products with different PC/PE ratios can be obtained. Products with a PC:PE ratio of >5:1 were obtained with 90% ethanol (Van Nieuwenhuyzen, 1976). Lecithin preparations of 95% PC and almost pure PC were obtained from soybean and egg lecithin, respectively, with an enzymatic method developed by Juneja et al. (1989). Chemical treatments have also been used for this purpose. Aneja and co-workers (1971) prepared PE-free phospholipids from soybean lecithin by acylation of AI.

Supercritical Fluid Extraction (SCFE) of oil from crude lecithin is a relatively new approach. Light-yellow colored, solid, de-oiled lecithin was obtained from crude soybean lecithin with SC-CO_2 extraction (Heigel and Hueschens, 1983). However, SCFE of phospholipids from oil seeds or fractionation of phospholipid mixtures using SCFE has not been reported. SC-CO_2 extraction of PL requires addition of a polar co-solvent to the supercritical phase since PL are relatively polar compounds which are sparingly soluble in SC-CO_2 (Friedrich and Pryde, 1984; Fattori et al., 1987, Temelli, 1992).

Although, modification of high erucic acid rapeseed lecithin has been studied (Niewiadomski, 1970), the improvement of lecithin quality from low erucic acid varieties (i.e. canola) has not been reported. Our objective was to modify crude canola lecithin for potential food applications. In this work, acetone refining, ethanol fractionation and SC-CO_2/ethanol mixtures were investigated to provide products with enriched PC levels from crude canola lecithin. SC-CO_2 extraction was included for comparison with conventional techniques to study its potential to produce PC of higher purity without organic solvents for pharmaceutical and food purposes.

MATERIALS & METHODS

CRUDE CANOLA LECITHIN was obtained from Canamera Inc., Fort Saskatchewan, AB, and stored below −20°C (after flushing the headspace with nitrogen). Acetone was (Omni Solv) 99.5% purity and ethanol (Commercial Alcohol Ltd, Montreal) was 99.9% purity. Hexane insoluble matter, moisture and AI contents of crude canola lecithin were determined according to the AOCS Official methods Ja 3-87, Ja 2-46 and Ja 4-46, respectively (AOCS, 1990). Averages of at least triplicate analysis were reported.

Phosphorus content was determined by perchloric acid digestion followed by spectrophotometric quantitation according to the Bartlett (1959) method as modified by Marinetti (1962). Conversion factor 25.6 was used to convert total phosphorus to PL amounts. Calculation of the conversion factor was based on the PL composition of canola AI reported by Vaisey-Genser and Eskin (1987). Molecular weights of individual PL components were from Fattori et al. (1987).

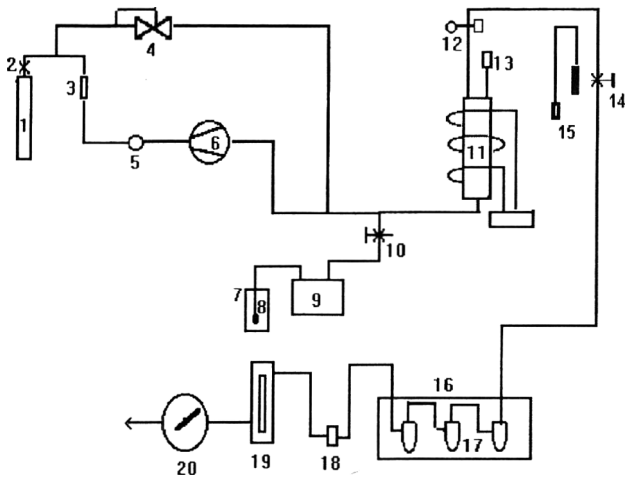
Acetone extraction of crude lecithin

AI were separated from crude canola lecithin by treating 50 g crude lecithin with 300 mL acetone followed by three extraction steps using 200 mL acetone in each. Extraction time was 1 h for each step with continuous stirring at 20°C. Each extraction step was followed by separation of acetone solubles from AI by decanting. Acetone was removed from the mixture of acetone solubles in a rotary evaporator and the yield of acetone solubles was determined gravimetrically. AI were dried under nitrogen and weighed.

Extraction times of 30, 60 and 90 min for each extraction step and 1–4 extraction steps were chosen to study the effect of extraction time on the acetone soluble yield at 20°C. Extractions were performed at 20, 35, 50°C and the duration of each extraction step was 60 min.

Acetone to crude canola lecithin ratio was varied as 1.6, 3.1 and 4.7 (w/w). These experiments were performed at 20°C as two extraction steps of 60 min each. Analysis of variance was conducted to determine the significance of the effects of the above variables at $p<0.05$ level.

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1-CO2 tank	8-filter	
2-valve	9-ethanol pump	15- temperature controller
3-CO2 filter	10-valve	16-cooling bath
4-pressure regulator	11-extractor	17-collection tubes
5-pressure gauge	12-extractor temperature controller	18-silica trap
6-compressor	13-rupture disc	19-flow indicator
7-ethanol reservoir	14- depressurization valve	20-flow totalizer

Fig. 1—Flow diagram of the SCFE unit. 1-CO_2 tank; 2-valve; 3-CO_2 filter; 4-pressure regulator; 5-pressure gauge; 6-compressor; 7-ethanol reservoir; 8-filter; 9-ethanol pump; 10-valve; 11-extractor; 12-extractor temperature controller; 13-rupture disc; 14-depressurization valve; 15-temperature controller; 16-cooling bath; 17-collection tubes; 18-silica trap; 19-flow indicator; 20-flow totalizer.

Table 1—Composition of crude canola lecithin

Moisture (wt%)	39.79 ± 0.43%
Hexane insolubles (wt%)	0.35 ± 0.05%
Acetone insolubles (wt%)	36.55 ± 0.50%
Total phosphorus (wt%)	1.83 ± 0.14%
Total phospholipids (P × 25.6)	46.85 ± 3.62%

Ethanol extraction of AI

AI were fractionated with ethanol according to the procedure outlined by Sholfield et al. (1950). The amount of ethanol used for fractionation was varied as 2.4 and 3.2 times the AI amount (w/w). Six extraction steps with each level of ethanol were carried out. Weights of extract and residue were determined after removal of solvent under nitrogen at 50°C.

Supercritical fluid extraction of AI

AI used for SCFE experiments, was obtained by treating 25 g of crude canola lecithin with acetone at room temperature (≈23°C). Oil was removed from crude lecithin in three consecutive extraction steps with 150, 100 and 100 mL acetone and 60 min of constant stirring for each step. AI was filtered out after each step and pooled AI was dried in a vacuum oven at 40°C for 24 hr. Dried samples were stored below 0°C. Total phospholipid contents of the samples were determined as described.

A laboratory scale SCFE unit (Newport Scientific Inc., Jessup, MD) used for fractionation of AI was described previously by Temelli (1992). This unit was modified for co-solvent injection into the supercritical solvent (99.9% CO_2, Medigas Alberta Ltd., Edmonton, AB) (Fig. 1). A high pressure pump (Gilson piston pump, model 305, Middleton, WI), capable of operating in flow, dispense or program modes, was used for co-solvent injection. Co-solvent ethanol was pumped into the system in the dispense mode (fixed volume at a specified rate) and/or in the flow mode (constant flow rate).

All SCFE experiments were performed at 55.2 MPa and 70°C. The extractor cell was loaded with 20g of canola AI for each experiment. SC-CO_2 and ethanol were added to the system in the following two stages: (1) **Pre-extraction period:** CO_2 was pumped into the system until the extraction conditions of 55.2 MPa and 70°C were stabilized. During pressurization, 0, 20, 40 or 60 mL of ethanol was pumped in the dispense mode over 2 min. Then, the compressor was continued for 1 h to improve mixing of CO_2 and ethanol. This period was referred to as "soaking" during which the depressurization valve was closed. (2) **Extraction period:** Extraction was started by opening the depressurization valve and continued for 3 hr during which a CO_2 flow rate of 1.3 ± 0.2 L/min. (at room temperature and pressure) was maintained. Throughout extraction ethanol was mixed into CO_2 in the flow mode at constant flow rate to give 13 ± 2% (mole %) of ethanol in CO_2. Extract and ethanol mixture were collected in glass tubes attached to the depressurization valve and placed in a refrigerated circulator bath (Lauda, model super RMT-6) at −15°C. Amount of extract was determined gravimetrically after removal of ethanol by a rotary evaporator. All experiments were performed at least in duplicate.

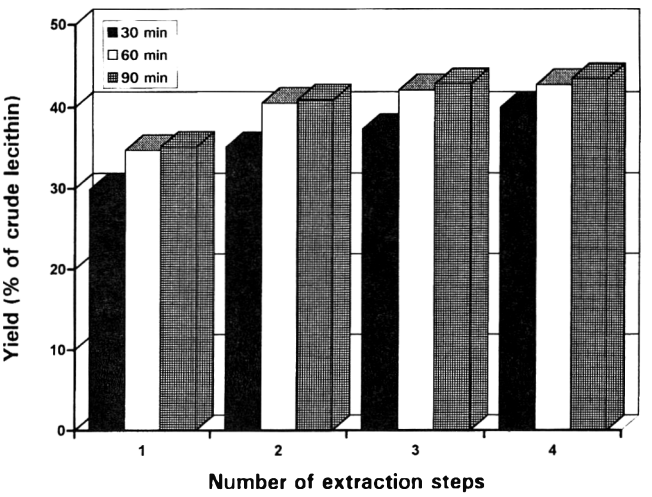


Fig. 2—Effect of extraction time on the yield of acetone solubles at 20°C. Amount of ethanol used for the extraction was 300 mL and 200 mL for first and consecutive extraction steps, respectively for all experiments.

Phospholipid analysis

Phospholipids in extracts were analyzed by an Iatroscan TH-10 Mark II analyzer (T.M.A. Scientific Supply, Mississauga, ON) equipped with precoated silica gel chromarods SII. Analysis was as described by Ratnayake and Ackman (1985). The chromarods were developed in two steps; first in acetone to remove nonpolar compounds, then in chloroform:methanol:water (65:35:4 v/v/v) to separate polar lipids. Chromarods were scanned under the following conditions: hydrogen pressure 0.75 kg/cm^2, air flow rate 2000 mL/min. Peak areas were integrated with the Hewlett-Packard Series 3356 (Hewlett-Packard, Avondale, PA) laboratory automation system. Phospholipid standards, PC, PE, PI and phosphatidic acid (PA) (Sigma Chemical Co., St. Louis, MO) were used for peak identification. Analyses were performed at least in duplicate.

RESULTS & DISCUSSION

Fractionation of crude canola lecithin with acetone and ethanol

Crude canola lecithin was characterized (Table 1). AI matter and phosphorus contents of crude canola lecithin were lower than those of rapeseed lecithin reported by Niewiadomski (1970), 36.55, 1.83%; and 55.9, 2.4%, respectively. Extraction parameters; time, temperature, solvent-to-feed ratio and the number of extraction steps were studied to maximize yield of acetone solubles. Acetone-benzene mixture was shown to extract more oil from crude rapeseed lecithin than did pure acetone (Niewiadomski, 1970). However, in our study, pure acetone was used since it is the solvent used for industrial applications. The yield of acetone solubles was related to the number of extraction steps and the duration of each step (Fig. 2). There was an increase ($p<0.05$) in the amount of acetone solubles when the time of each extraction step was increased from 30 min to 60 min, but effects of further increases to 90 min were not significant. Two extraction steps of 60 min each with 300 and 200

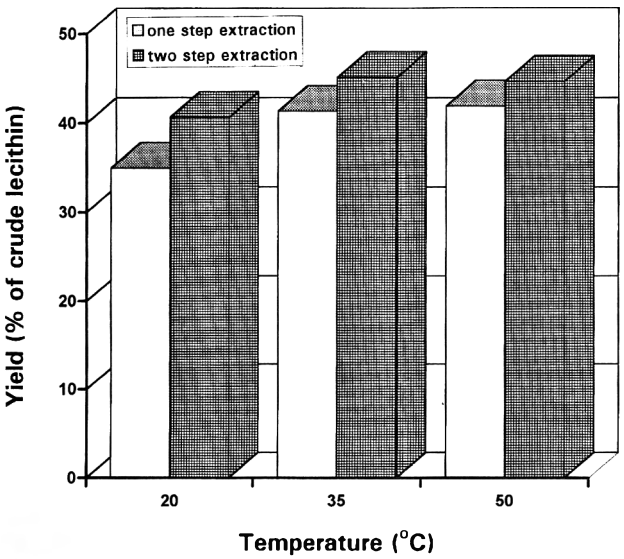


Fig. 3—Effect of extraction temperature on yield of acetone solubles.

mL acetone was adequate since the yield of acetone solubles reached a plateau in that time. Under these conditions 40.5% of the crude lecithin was extracted with acetone (Fig. 2). Niewiadomski (1970) showed that although the amount of acetone soluble compounds decreased with increasing numbers of extractions, phospholipid concentration in the acetone soluble fraction of rapeseed lecithin increased. Therefore, it is essential to keep the number of extraction steps for removal of neutral lipids at an optimum level to minimize loss of phospholipids with acetone.

Temperature also affected extraction yield (Fig. 3). The optimum temperature was 35°C, since at 20°C there was a decrease ($p<0.05$) in the amount of acetone solubles and increasing to 50°C did not result in a significant ($p>0.05$) increase in yield. Two extractions of 60 min each at 35°C using a total of 500 mL of acetone were optimum conditions at which 34% of crude lecithin was recovered as AI. This showed that only 2.55% of the AI in crude lecithin was lost due to higher extraction temperature (35°C) compared to the official method of AI determination which was performed at 5°C (Table 1).

As the acetone:crude lecithin ratio increased from 1.6 to 4.7 (w/w), yield of acetone solubles also increased. The highest yield of acetone solubles (28.16%) was obtained with acetone: crude lecithin ratio of 4.7, while the yield at a ratio of 1.6 was only 18.88% after 60 min extraction.

AI obtained under optimum conditions was further fractionated using ethanol. Increasing the ethanol:AI ratio from 2.4 to 3.2 (w/w) had no significant ($p>0.05$) effect on yield of ethanol soluble and insoluble fractions. AI were fractionated into 68.7% and 26.5% ethanol insoluble and soluble fractions, respectively. When a similar experiment was performed with defatted rapeseed lecithin, a higher ethanol insoluble fraction yield of 75.8% was reported by Niewiadomski (1970).

Total PL content of lecithin fractions were compared (Table 2). Acetone extraction at optimum conditions (35°C and two steps of 60 min each) gave the highest PL concentration in the AI. Under these conditions PL content was increased from 46.85% in crude lecithin to 66.7% in the AI. Further fractionation of AI at an ethanol:AI ratio of 3.2 (w/w) resulted in 68.4% and 71.5% PL in ethanol solubles and insolubles, respectively.

Fractionation of AI with SC-CO_2/ethanol mixture

Preliminary results of the fractionation of canola AI with SC-CO_2/ethanol mixtures were compared (Table 3). Results showed that 0.3–0.5 g of ethanol free extract was collected during 3 hr

Table 2—Phospholipid content of the ecithin fractions from different extraction conditions.

Sample	Temperature (°C)	Time (min)	PL (wt%)
Acetone insolubles	20	30	58.9
	20	60	61.7
	20	90	63.3
	35	60	66.7
	50	60	62.9
Sample	**Ethanol/AI ratio (w/w)**		**PL**
Ethanol solubles	2.4		61.1
	3.2		68.4
Ethanol insolubles	2.4		59.6
	3.2		71.5

Table 3—Amount and composition of canola AI fraction extracted with SC-CO_2 and 13±2 mole % ethanol at 55.2 MPa/70°C.

Ethanol for soaking (mL)	0	20	40	60
Amount of extract (g)	0.3	0.3	0.4	0.5
Total PL in extract (wt%)	6	9	26	32
PC content of PL in extract (Iatroscan area %)	48	73	86	83

of extraction at 55.2 MPa and 70°C corresponding to quite low extraction yields of only 1.5–2.5%. These extraction conditions were chosen because previous experiments performed with flaked canola seeds and SC-CO_2/ethanol mixture gave highest PL concentration under those conditions (Dunford and Temelli, 1994). Total phospholipid concentration of the fraction extracted using SC-CO_2/ethanol (13 mole %) solvent mixture without soaking was 6%, very low for any practical use of such an extract as an emulsifier. In an effort to increase the amount of extract and its phospholipid concentration, crude lecithin samples were soaked with ethanol under high pressure prior to extraction. Such practice has been common in SC-CO_2 extraction of caffeine from coffee beans where the beans and solvent must be saturated with water (Zosel, 1978). As the amount of ethanol for soaking was increased, the amount of extract and its phospholipid concentration increased from 0.3g to 0.5g and 6% to 32%, respectively. However, the efficiency of the process was still low compared to that from ethanol alone. AI formed a hard cake in the extractor under high pressure and in the presence of liquid ethanol without mixing. One reason for low extraction efficiency would be the reduced CO_2 contact with AI due to cake formation. Mixing the cell contents during extraction or use of a more polar entrainer such as methanol might improve extraction efficiency. For our purposes, mixing was not possible due to limitations of our system and use of methanol was avoided due to food application objectives.

PC content of the extracts

Since one objective was to obtain PC enriched fractions, extracts were analyzed for their PC contents. We could not obtain a clear separation of PL components other than PC by the Iatroscan method, therefore peak areas for all PL components other than PC were pooled for PC concentration calculation. PC percentages of the samples were reported as the Iatroscan area % of the total PL present in the samples (Tables 3 and 4). Acetone extraction did not appreciably affect PC content of samples. PC content of the AI obtained under optimum conditions was 40.8%. Fractionation of AI with ethanol at ethanol/AI ratio of 2.4 (w/w) yielded ethanol soluble and insoluble fractions with 85.2 and 14.9% PC, respectively. Ethanol/AI ratio for extraction apparently did not affect PC concentration of the extracts. Analysis of the extracts from the SC-CO_2/ethanol mixture showed that soaking of AI samples prior to extraction increased PC content of the extracts. While extracts obtained without soaking had only 48% PC, those obtained after soaking with 20 mL ethanol had PC concentration of 73%. Maximum PC content of 86%

Table 4—PC content of the PL in canola lecithin fractions

Sample	PC (Iatroscan area %)
Crude lecithin	39.6
AI (35°C)	40.8

Sample	Ethanol/AI ratio (w/w)	PC (Iatroscan area %)
Ethanol solubles	2.4	85.2
	3.2	84.3
Ethanol insolubles	2.4	14.9
	3.2	14.6

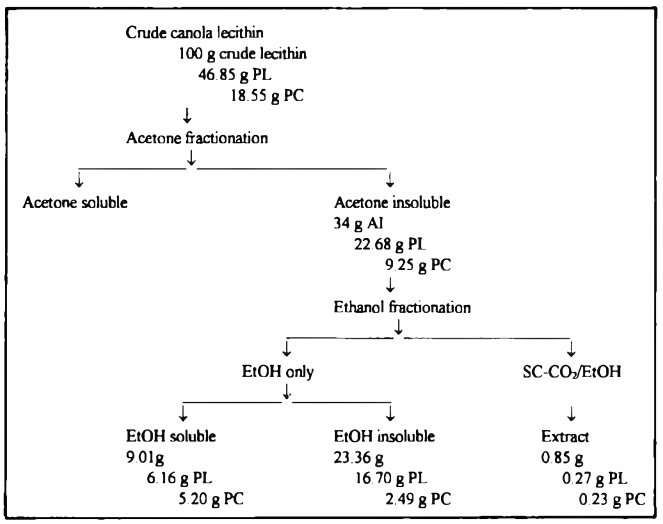


Fig. 4—Material balance for fractions of crude canola lecithin.

was achieved with 40 mL ethanol soaking. Further increase of ethanol used for soaking to 60 mL did not affect PC percentages ($p>0.05$).

Material balances were calculated for all fractions under optimum conditions (Fig. 4) based on 100 g crude lecithin starting material containing 46.85 g PL, of which 18.55 g was PC. This calculation assumed the Iatroscan area % for PC to be equivalent to the wt % in the PL mixture. Fractionation of AI with ethanol resulted in PC recovery of 28.0 and 13.4% in crude lecithin in the ethanol soluble and insoluble fractions, respectively. PC recovery by the SC-CO_2/ethanol mixture was very low (1.2% of original PC).

CONCLUSIONS

TWO EXTRACTION STEPS of 60 min each at 35°C using a total 500 mL of acetone were optimum conditions to maximize removal of acetone solubles from crude canola lecithin. These conditions also gave maximum PL concentration, 66.7%, in the acetone insolubles Ethanol extraction of AI using an ethanol: AI ratio of 3.2 (w/w) gave the highest PL concentrations. Modification of crude canola lecithin with acetone and ethanol extraction resulted in an increase in PL content from 46.85% to 71.5%. Initial soaking of AI with ethanol was necessary to achieve 32% PL concentration in the SC-CO_2/ethanol extract of AI. Extracts with PC concentration >85% could be obtained by both acetone/ethanol and SC-CO_2/ethanol extraction. However, the very low yields obtained under supercritical conditions indicate that high pressure SCFE of phospholipids would not likely be economically feasible. Design of a supercritical extractor with a mixing mechanism for better contact between solvent and feed should improve yields. Canola lecithin shows potential for obtaining a high-value PC enriched fraction for specific food or pharmaceutical applications.

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Biologically Active Components Inactivation and Protein Insolubilization during Heat Processing of Soybeans

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ABSTRACT

Inactivation of lipoxygenase, trypsin inhibitor, urease and retention of protein solubility during water blanching of dehulled soybeans at 90°, 95° and 100°C were investigated. Lipoxygenase was the most heat labile, followed by urease and then trypsin inhibitor. Processing time based on "acceptable inactivation time" (AI) was proposed. AI value was longest for trypsin inhibitor, followed by urease and then lipoxygenase. The combined effect of heat on protein solubility and biologically active components inactivation was expressed as "PDI at acceptable inactivation time" (PDIAI). PDIAI value for the processing "limiting factor," trypsin inhibitor inactivation, at the three temperatures were 36.7%, 42.5%, and 34.8%, respectively.

Key Words: soybeans, lipoxygenase, trypsin inhibitor, urease, protein solubility

INTRODUCTION

PROCESSING OF SOYBEANS, typically by heat treatment, improves nutritional value through conversion of native (refractory) proteins into more digestible denatured forms (Boonvisut and Whitaker, 1976). It also destroys heat-labile biologically active components, such as trypsin inhibitor, urease, hemagglutinins, allergins, saponins and goitrogens (Rackis, 1974). Trypsin inhibitor, urease, and lipoxygenase are used as indices of adequate processing for edible soy products (Rackis et al., 1975b). Trypsin inhibitor accounts for 30–50% of the growth inhibitory effect and nearly all of the pancreatic hypertrophic response of animals fed raw soybean meal (Rackis, 1980). Urease enzyme seems to serve no practical physiological function in humans, but can produce toxic effects in animals fed raw soymeal. Urease is relatively heat stable and its analysis is simple. Thus, its inactivation is used by various soybean processors as an index for determining the degree of heat treatment received and quality of various soy products. Typically, lack of urease activity indicates adequate trypsin inhibitor inactivation.

The flavor of soy products is a major quality concern. Lipoxygenase enzyme activity results in rancidity, poor storage stability, and painty, grassy, or beany off-odors and off-flavors. Hydroperoxides formed by lipoxygenase also affect: color, by a Maillard type reaction interacting with proteins; the texture by cross-linking with proteins; and the nutritive value by a decrease of essential fatty acids and certain vitamins (Eriksson, 1982). Secondary products arising from hydroperoxide decomposition also readily inactivate or denature protein and amino acids through formation of covalent bonds (Gardner, 1979).

Although thermal treatment of soybeans is necessary, overheating will cause a loss in nutritional value by reducing the protein efficiency ratio (PER). This is due to destruction or rendering of several essential amino acids unavailable, such as cystine and lysine (Del Valle, 1981). In addition, overheating adversely affects the functionality (versatility) of soy in a food system. Solubility profile of proteins is proposed to be a good indicator of functionality (Mattil, 1971). Solubility directly relates to many other important functional properties such as emulsifying capacity and stability (Volkert and Klein, 1979), foaming, gelation, and viscosity.

An optimal thermal process inactivates deleterious enzymes, microbes and biologically active components, while maximizing retention of nutrients and other quality attributes (flavor, color, texture, functionality). Severity of heat treatment to destroy biologically active components is considerably less than that leading to impaired or decreased protein nutritional quality (Del Valle, 1981). Thermal processes should be based on inactivation of the most heat-resistant factor, referred to as the "limiting factor" of the process.

Heat processing of soybeans need not be based on total deleterious components inactivation. Labuza (1973), converting the data of Hackler et al. (1965) into approximate kinetic parameters, pointed out that doubling the rate of trypsin inhibitor destruction by increasing process temperature would increase the destruction of many nutrients four- to fivefold. In most cases it is not necessary to reach complete inactivation. Thus, kinetic data are needed to maximize destruction of deleterious components while minimizing loss of essential nutrients.

Biologically active components inactivation has been well documented. However, few studies have focused on a kinetic model for quantitatively defining the heat lability of these components relative to one another. Thus, our objectives were to: investigate thermal inactivation kinetics of biologically active components, and determine the effects of temperature on biologically active components inactivation combined with protein solubility changes.

MATERIALS & METHODS

Soybeans

Williams 82 variety soybeans were grown on farm plots at the University of Illinois. The harvested beans were cleaned, dried at ambient temperature (~25°C) to about 12% moisture in a variable circulation drier, and stored at −17.8°C. Dehulled soybeans were prepared by the method of Shyeh et al. (1980) and stored at −17.8°C until heat processed.

Heat processing

For each sample, 200g of dehulled soy cotyledons were placed in a wire mesh basket, and lowered into a heating medium of 1:20 (bean: water ratio) at 90°, 95°, or 100°C for 5, 10, or 15 min. After heating, the basket with sample was removed and placed immediately in an ice/water bath for about 1 min. Samples were then frozen overnight at −40°C, followed by freeze drying (at room temperature, ~23°C; pressure of 10 microns) for 3 days. Samples were then stored at −28.9°C until chemical analysis was carried out. A control (no heat treatment) was prepared by subjecting 200g of untreated dehulled soybeans to the same freeze drying conditions.

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Table 1—Kinetic data for biologically active components inactivation: (dehul ed) Williams soybeans

Factor	Temp (°C)	k (min^{-1})	D (min)	E_a (kcal/m)	z (°F)
LO	90	0.430	5.3	31.8	z_5 = 34.1
	95	0.795	2.9		
TI	90	0.111	20.8	11.5	z_5 = 64.6
	95	0.153	15.1		z_{10} = 97.2
	100	0.170	13.5		
U	90	0.250	9.3	21.1	z_5 = 65.5
	95	0.344	6.7		z_{10} = 52.6
	100	0.550	4.2		

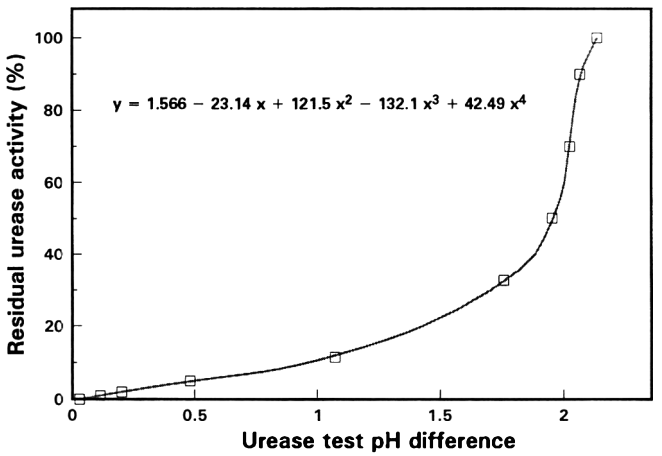


Fig. 1—Residual urease conversion curve for dehulled Williams soybeans.

Protein content and solubility

Nitrogen content was determined by micro Kjeldahl, AOCS Method Aa 5-38 (AOCS, 1984). Protein content was calculated by multiplying by 6.25. Dispersible protein was determined by the "fast-stir" method for protein dispersibility index (PDI) according to AOCS Method Ba 10-65 (AOCS, 1984).

Oil extraction

Defatted samples for trypsin inhibitor and lipoxygenase analysis were prepared by extracting oil from ground soybeans with petroleum ether according to AOCS Method Aa 4-38 (AOCS, 1984). Residual solvent was removed by vacuum without heat, and the sample stored at −28.9°C.

Biologically active components assays

The spectrophotometric method for lipoxygenase enzyme (L-1 isoenzyme) assay developed by Surrey (1964) and modified by Lao (1971) was used to follow the rate of conjugated diene formation at 234 nm. Trypsin inhibitor content was determined according to AOCS Method Ba 12-75 (AOCS, 1984), with modification by Hamerstrand et al. (1981). Urease enzyme activity was determined by AOCS Method Ba 9-58 (AOCS, 1984).

RESULTS & DISCUSSION

Biologically active components inactivation

Mean values for unprocessed control (no heat treatment) dehulled Williams soybeans were: lipoxygenase: 16.8 ± 1.25 LA/mg solid; trypsin inhibitor: 21.3 ± 0.08 TI mg/g; and urease: 2.13 ± 0.052 pH increase. Lipoxygenase activity for dehulled soybeans was lower than for unprocessed whole Williams soybeans (21.8 ± 0.55 LA/mg solid). The dehulling process (which incorporates heating whole beans for 15 min at 98.9°C) resulted in 23% loss of initial lipoxygenase activity. Losses of initial trypsin inhibitor and urease activities with dehulled samples were insignificant, indicating that lipoxygenase was more heat sensitive than trypsin inhibitor or urease.

For a given temperature, the log biologically active component activity-processing time relationship was determined with a line fit to the data using the least squares criterion (Box et al., 1978). This fitted line was defined as follows:

$$\hat{Y}_0 = b + m\,T_0 \qquad (1)$$

where $\hat{Y}_0$ is the predicted log activity corresponding to a given blanching time T_0, m is the slope, and b is the y-intercept. This line allowed for calculation of various parameters from standard thermal processing equations (Lund, 1975). The calculated kinetic parameters: reaction rate constant (k), D value, activation energy (E_a), and z value, for lipoxygenase (LO), trypsin inhibitor (TI), and urease (U) inactivation for dehulled Williams cotyledons were compared (Table 1). Results of LO inactivation at 100°C were not included because complete inactivation occurred before the first sample processing time (5 min).

For urease inactivation, results of the assay provide an index of enzyme activity, but a linear relationship between the enzyme present and the pH reading of the urease assay should not be assumed. To provide a common basis for comparison, Baker and Mustakas (1973) converted urease pH difference values to percent residual urease activity. They used blends in varying proportions of raw soy flour in which urease was intact with an extrusion-cooked soy flour where the urease had been totally destroyed. The pH increase for blends was then plotted vs. the associated percent residual activity.

This curve was developed for our study using blends in varying proportions of raw dehulled Williams soybeans with dehulled soybeans heated at 100°C for 45 min to represent 100% and 0% activity, respectively. Comparing this urease conversion curve for dehulled soybeans (Fig. 1) to that of Baker and Mustakas (1973), in the range of 0–30% residual urease activity, the curve generated for our study had a steeper slope. This suggested that a curve to convert urease data from pH difference to percent urease activity (%U) should be made for each type soybean sample studied. A fourth order regression polynomial equation (Fig. 1) was used to convert all urease data from pH difference to %U. This data was then utilized to determine k, E_a, D and z values for urease inactivation (Table 1).

Urease inactivation data exhibited a significant difference in %U determined from pH difference compared to that obtained using a residual activity conversion curve. Data points for dehulled Williams, blanched at 95°C for %U from pH difference, were compared with that using the conversion curve. %U values were: at 5 min blanching, 72.0% (pH difference) vs 19.2% (conversion curve); at 10 min, 19.8% vs 4.4%; and at 15 min, 4.1% vs 0.7%, respectively (Savage, 1989). For each of the three temperatures, %U values were higher when the residual conversion curve was not used.

If the higher values (determined from pH difference) were plotted and compared to TI or other biologically active components inactivation, the urease curve might be above that of these other factors, implying inaccurate heat lability conclusions. Published findings which have not taken this factor into consideration may have made erroneous comparisons which may affect the reliability of using urease inactivation as an indicator for other biologically active components inactivations. If %U data is not obtained, the urease assay would not be an accurate indication of urease inactivation and could lead to overprocessing.

The reaction rate constant data were consistent with reported results. After converting data of Wapinski (1977) for dehulled Bonus soybeans at 19.4% moisture into kinetic results, a k value for LO of 0.305 min^{-1} was calculated for immersion heating at 82°C. In addition, converting data for dehulled samples at 9.5% moisture steamed at 98.9°C resulted in a k value of 0.9815 min^{-1}. For TI, k values were consistent with calculated values converted from data of McNaughton et al. (1981) with autoclaved soybean meal (k = 0.192 min^{-1}) and from Soertrismo et

al. (1982) with autoclaved soaked soybeans (k = 0.195 min^{-1}). For U, converting pH difference data of McNaughton et al. (1981) resulted in a k value of 0.225 min^{-1}. This was comparable to results of our study, if basing inactivation at 100°C on pH difference data (k = 0.292 min^{-1}) and not using the residual conversion curve concept.

For all three biologically active components, the reaction rate constants increased as blanching temperature increased. This was expected since more thermal energy is being transferred as blanching temperature is increased. The greatest reaction rate of inactivation at each temperature occurred with LO, followed by U and TI. This indicated that of the three factors studied, LO was the most heat labile and TI was the most heat stable.

The temperature dependence of kinetics or heat sensitivity of biologically active components could be seen by the difference in k values as blanching temperature increased. As the temperature increased from 90° to 95°C, the k values for LO, TI and U increased by 84.9%, 37.8%, and 37.6%, respectively. An increase in temperature increases the average kinetic energy and average velocities of molecules in the reaction, which allows for greater rates of inactivation.

At 90°C, D values for LO, TI, and U (5.3, 20.8, and 9.3 min, respectively) indicated that 90% inactivation for each factor compared to LO would require almost two times longer to inactivate U, and four times longer for TI inactivation. As blanching temperature increased, the D value of each factor decreased, again illustrating the benefit of increased blanching temperature. At 95°C, D values indicated that in comparison to LO, approximately two times longer was required to inactivate U, and five times longer for TI. This indicated the greater heat lability of LO, compared to TI or U, as the temperature was increased.

A denaturation reaction characterized by low activation energy (such as E_a = 20 kcal/mole; for peroxidase in peas) indicates that the active site of the compound is dependent on only a few bonds (Lund, 1975). For LO the E_a is on the order of breaking of a disulfide bond (E_a = of 30 kcal/mole), whereas the E_a for TI is on the order of two H bonds (E_a = 5 kcal/mole). Farkas and Goldblith (1962) studying the inactivation of a stock LO solution using heat and ionization radiation, obtained a E_a of 101 kcal/mole. Addition of 20% pea solids afforded a 6- to 10-fold protection with respect to heat inactivation. This illustrated that to gain complete understanding of inactivation, real food systems need to be investigated. The thermal stability of several enzymes would be affected by the presence of specific substrates or coenzymes. In the soybean, natural components occur which may affect overall activity, and heat penetration would be lower in the solid bean system vs. pure enzyme analysis.

The E_a values for TI are consistent with data reported by Johnson et al. (1980) E_a = 11.3 kcal/mole, and converting heat treatment data of soymilk samples prepared by Hackler et al. (1965) E_a = 18.6 kcal/mole and Van Buren et al. (1964) E_a = 11.1 kcal/mole. For U, Sizer (1940) obtained a E_a value of 11.0 kcal/mole with a purified enzyme solution from soy flour. The addition of soy solids again affected the overall inactivation. Comparing the E_a for the three biologically active components, LO showed the most heat sensitivity followed by U and then TI.

The z value for LO in the range 90–95°C was half that of U or TI. This indicated that LO was much more temperature dependent between 90–95°C than TI or U. Although LO per se is very active (based on its high turnover number of 2×10^4 mol/mol enzyme/min) it is also easily inactivated. It is important to eliminate the action of LO by adequately processing soybeans. If flavor problems occur after processing, these are probably due to other factors, such as peroxidase. In the range of 90-100°C, TI became more temperature independent than urease. This meant that increasing the processing temperature to 100°C was more important in the inactivation of U than for TI.

From the comparison of kinetic values for all three biologically active components, the reaction rate (k) was lowest for TI and changes in processing temperature (z value) had the least effect on TI. Thus, TI could be considered the most important biologically active component ("limiting factor") for controlling the blanching process for dehulled Williams soybeans. Use of the urease assay to determine adequate heat processing of dehulled Williams soybeans would result in underblanching with respect to TI and other undesirable results. Even by conversion of pH increase data to %U concentration, the reaction rate plot contained wide deviations from linear inactivation (which affected the variance, and subsequent determined AI value), particularly with longer processing times and as temperature of blanching increased. Therefore, for adequate process control, emphasis should be placed on TI inactivation rather than on U.

Acceptable inactivation of biologically active components

The kinetic inactivation reaction rate data for each biologically active component was used to calculate its maximum acceptable residual activity. We developed the concept of "acceptable inactivation time" (AI), which denotes the critical blanching time required to result in desired residual biologically active component activity. This concept more precisely defines the extent of inactivation for controlling blanching. AI value takes into account the physical nature of the sample, the initial biologically active component concentration, and the percent inactivation required.

An algorithm was developed using the fitted inactivation rate for each biologically active component (Eq. 1) to determine this critical blanching time. The acceptable inactivation of the biologically active components (which will be denoted Y_{crit}) was determined, based upon the initial biologically active component concentration (A_0), and the degree of inactivation (I) required. Rackis et al. (1975a) observed that only 50–60% inactivation of TI was needed to eliminate pancreatic hypertrophy in rats, with 70–80% inactivation resulting in maximum PER for a diet containing soy flour. For urease inactivation, a urease assay pH increase of less than 0.15 is considered sufficient, based on adequate heat treatment for animal feed and edible soy flours (McNaughton et al., 1981). Soybean processing plants typically use a urease index of 0.2 pH increase (which corresponds to 90% inactivation) to indicate adequate heat processing (Wright, 1981). The elimination of most off-flavors requires almost complete inactivation of lipoxygenase enzyme. Thus, the degree of inactivation (I) required were: trypsin inhibitor (I = 80%), urease (I = 90%), and lipoxygenase (I = 99%). The acceptable inactivation Y_{crit}, was determined as follows:

$$Y_{crit} = \log \frac{A_0 \cdot (100 - I)}{100} \quad (2)$$

The blanching time corresponding to Y_{crit} was found as follows:

$$T^* = \frac{Y_{crit} - b}{m} \quad (3)$$

where T* is the blanching time required for adequate biologically active component inactivation. Y_{crit} may be viewed as the mean log activity produced by a blanching time of T* (Fig. 2). To reflect the uncertainty in the least squares estimates (b and m), on predicted log activity, a confidence interval for the true mean log activity was developed. The confidence interval indicates that 95% of future mean log activities would fall within this interval (Huntsberger and Billingsley, 1981).

Since the primary concern is individual activity and not the mean activity, a 95% prediction interval was developed for the fitted line. The interpretation of such a prediction interval indicates that 95% of future activities would lie within this interval (Huntsberger and Billingsley, 1981). The 95% prediction interval is given by:

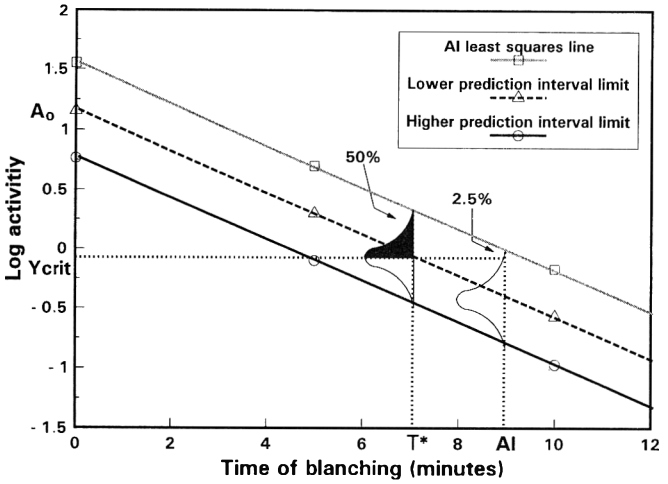


Fig. 2—Illustration of acceptable inactivation time (AI) value determination by use of the biologically active component inactivation least squares line.

$$\hat{Y}_0 \pm t_{\alpha/2,\ n-2} S_{Y0} \tag{4}$$

where S_{Y0}, the standard error associated with a future trial is:

$$S_{Y_0} = S_r \left[1 + \frac{1}{n} + \frac{(T_0 - \bar{T})^2}{\sum_{i=1}^{n} (T_i - \bar{T})^2} \right]^{1/2} \tag{5}$$

where T_i = time for the i th trial; $\bar{T} = \sum_{i=1}^{n} T_i/n$, the mean time of the trials; n = number of sample trials used to develop the model; S_r = the standard error associated with the model residuals.

If several samples were processed with a time T*, ~50% of the resulting sample activity concentrations would lie above Y_{crit} (Fig. 2). Almost all of the measured future activities should be less than Y_{crit}. Thus to ensure adequate blanching, a blanching time should be used where the upper limit of the biologically active component activity prediction interval is equal to Y_{crit}. For this blanching time, which we are defining "acceptable inactivation time" (AI), only 2.5% of future measured activities would be above Y_{crit}. The upper limit of the prediction interval (equal to Y_{crit}) is related to the AI by:

$$Y_{crit} = b + m\,(AI) + t_{\alpha/2,\ n-2}\, S_r \left[1 + \frac{1}{n} + \frac{(AI - \bar{T})^2}{\sum_{i=1}^{n} (T_i - \bar{T})^2} \right]^{1/2} \tag{6}$$

The value that satifies Eq. (6), denoted as the AI value, was found by using a numerical Newton-Raphson root finding technique (Purcell, 1972).

The standard error of the residuals of each inactivation curve as well as the values for T*, and AI value for inactivation were determined (Table 2). For all three temperatures, the AI value for blanching was greatest for TI, followed by U and then TI. Raising the blanching temperature from 90° to 95°C decreased the AI value for LO by 5.9 min (process time decrease of 47.6%), for TI by 7.0 min (decrease of 38.5%) and for U by 4.0 min (decrease of 29.2%). As the temperature was increased from 95° to 100°C, AI value for U decreased by 2.3 min (decrease of 23.7%), while for TI it remained essentially the same. It would appear that increased temperature did not have much effect on TI activation. However, comparing the T* values for blanching at 95°C (10.4 min) and 100°C (8.7 min) showed that temperature still had an effect.

Table 2—Acceptable nactivation time (AI) value data for biologically active components: dehulled Williams soybeans

Factor	Temp (°C)	Rel std dev	T* (min)	AI (min)
LO	90	0.145	10.3	12.4
	95	0.075	5.8	6.5
TI	90	0.059	14.6	18.2
	95	0.019	10.4	11.2
	100	0.095	8.7	12.0
U	90	0.168	10.1	13.7
	95	0.189	6.9	9.7
	100	0.159	5.3	7.4

One possible explanation for absence of a temperature effect on AI value for TI as temperature was increased from 95° to 100°C, is in general, the effect of faster rates of inactivation at increased temperatures. When running several experiments with "equal" blanch times at 100°C, small differences in actual blanching times can produce wide differences in activities. Thus, at higher temperatures greater variability occurs in the biologically active components concentration values. The increased variability in such concentrations may be seen by the residual sample relative standard deviation (RSD) for the TI inactivation curve at 100°C (0.095) which was five times that which occurred at 95°C (0.019). The larger RSD at 100°C produced a wider prediction interval, which substantially increased the AI value. To reudce the amount of noise and subsequently demonstrate the expected effect of temperature on AI value, experimental parameters (such as blanching time and temperature) need to be more closely controlled to reduce the range of the prediction interval.

Large RSD values were also obtained for LO at 90°C and at all three temperatures for U. With LO, because of the large reaction rates in general, and specifically at that temperature, deviations from the curve will not have as much an effect on the calculated AI value. This is best seen by comparing T* and AI value for LO at 95°C (a difference of 0.7 min, RSD = 0.075) to T* and AI value for TI at 95°C (a difference of 0.8 min, RSD = 0.019). For U, the RSD for all three curves are the largest recorded. This indicated the difficulty in obtaining linear inactivation data even with converting pH difference data obtained with the urease assay into %U data.

The heat sensitivity of LO enzyme can be seen by comparing the calculated AI values. The decrease for AI value of LO as temperature is raised from 90° to 95°C was almost as great as increasing the temperature from 90° to 100°C for U (AI value decrease of = 6.3 min; which is a process time decrease of 46.0%) and TI (AI value decrease of 6.2 min; process time decrease of 34.1%).

It is important to compare AI value data (Table 2) to the D value (Table 1) for each biologically active component at each temperature. For LO at 90°C the AI value is 7.1 min longer than the D value; at 95°C the AI value is longer by 3.6 min. Therefore, if D values were used to monitor blanching, the product would be underblanched and effects on flavor may occur.

The differences between D value and AI value for TI at 90°, 95°, and 100°C were 2.6, 3.9, and 1.5 min, respectively. If blanching time was based on D value, the product would be overblanched by 2–4 min. Because trypsin inhibitor is the "limiting" biologically active component for blanching, this time difference could be important. While a difference of 2–4 min may not seem large, other detrimental effects on the product would occur due to overprocessing. In addition, use of the 95% prediction interval to determine the AI value incorporates a "safety factor" for processing. Use of the AI value insures acceptable biologically active components inactivation in 97.5% of all samples processed.

For urease, the differences between AI value and D value at 90°, 95°, and 100°C were 4.4, 3.0, and 3.1 min, respectively. Since D value is defined as the time which produces 90% inactivation, we anticipated that the AI value and D value for U

would be close. Reported differences may be due to the prediction interval concept for AI value, which considers inactivation of single samples, vs inactivation based on the mean activity as for calculating D value from the reaction rate curve. Comparison of "acceptable inactivation time" for each biologically active component inactivation at each temperature showed that the AI value for TI was consistently the highest. Thus, determination of adequate processing should be based on TI inactivation.

Protein insolubilization

The reaction rate plot of protein dispersibility index (PDI) was first order for the three temperatures studied, making it possible to determine rates of protein insolubilization. The kinetic data for PDI with 90°, 95°, and 100°C blanching were compared (Table 3). As temperature increased, protein insolubility increased as seen by both increasing k values, and decreasing D values. For all three blanching temperatures, the reaction rate of protein insolubilization was much lower (D values much higher) than for biologically active components inactivation (Table 1). The k value for protein insolubilization at 95°C was 8.1%, 42.0% and 18.8% that of the k value for LO, TI, and U inactivation, respectively. Thus protein solubility would be affected less by blanching than would biologically active components inactivation.

Comparing the D values of PDI (Table 3) vs biologically active components inactivation (Table 1), at 90°C the D value for PDI was nine times that of LO and five times that of U. As the temperature was raised to 95°C, the D value for PDI was 12 times that of LO and five times that of U. This indicated a greater rate of LO inactivation vs protein insolubilization. At 100°C, the D value for PDI is eight times that of U, indicating that U inactivation occurred much faster than the change in PDI. For TI inactivation at all three temperatures, the D values for PDI were two times that of TI inactivation. This indicated that the rate of both TI inactivation and protein insolubilization were affected to a similar extent as temperature was increased. The reaction rate of protein insolubilization was affected less by temperature than was biologically active components inactivation. For the temperature range 90–95°C, the z value was almost two times larger than that for LO inactivation, and slightly higher than for both TI or U. From 90–100°C, the z value was slightly higher than that for TI, and much larger than that for U.

PDI at acceptable inactivation

Using the AI value for each biologically active component, resulting protein solubility was calculated, based upon required processing time and rate of protein insolubilization. An algorithm was developed to determine the %PDI at the critical blanching time. Initially, the log PDI-time relationship was determined with a line fit to the kinetic data using the least square criterion (Table 3). Based upon a particular blanching temperature, and "acceptable inactivation time" (AI), the predicted %PDI response was determined. Results were referred to as "PDI at acceptable inactivation time" (PDIAI). Due to uncertainty in the least squares estimates, and the inherent variability in PDI response, a 95% prediction interval was determined to indicate that 95% of future trials conducted at a time equal to AI value would produce a PDI response lying within this interval.

The calculated values for the mean PDIAI value at each blanching temperature were determined (Table 4). The lowest PDIAI value was observed for TI, which was expected due to TI requiring the longest inactivation time. As blanching temperature increased, the PDIAI value for LO and U were favorably affected. As temperature is increased from 90° to 95°C for inactivating 99% LO, the PDIAI value increased 8.8%. When inactivating 90% U, as temperature increased from 90° to 100°C, the PDIAI value increased by 3.4%. The results with LO and U illustrate the desirable effects of increased temperature on functionality based on PDI, while at the same time accomplishing biologically active components inactivation.

Table 3—Kinetic data for protein insolubility: dehulled Williams soybeans

Temp (°C)	k (min^{-1})	D (min)	E_a (kcal/m)	z (°F)
90	0.048	47.7	11.3	z_5 = 72.0
95	0.064	35.8		
100	0.074	31.3		z_{10} = 95.7

Table 4—PDI at acceptable inactivation time (PDIAI) value data: dehulled Williams soybeans

Temp (°C)	Factor	AI (min)	mean PDIAI (%)	Prediction interval PDIAI (%)
90	LO	12.4	48.5	43.9–53.5
	TI	18.2	36.7	32.6–41.4
	U	13.7	45.6	41.1–50.5
95	LO	6.5	57.3	49.5–66.3
	TI	11.2	42.5	36.3–49.8
	U	9.7	46.6	40.0–54.3
100	TI	12.0	34.8	27.5–44.0
	U	7.4	49.0	39.2–61.3

For TI inactivation, the highest PDIAI value occurred for 95°C blanching. Blanching at 95°C was apparantly the preferable condition for TI inactivation, while at the same time causing acceptable inactivation of LO and U. Note, however, that the PDIAI value at 100°C is based on an AI value of 12.0 min. As previously mentioned this value may be inaccurate based on the standard deviation for this processing condition. In comparison, when PDIAI value for 100°C blanching was calculated based on the T* value (8.7 min), or by using the AI value for 95°C inactivation (11.2 min), the PDIAI values were 44.3% and 37.0%, respectively (Savage, 1989). Thus, for PDIAI value for TI inactivation at 100°C, we expected that the value would be closer to 44%, if experimental factors were controlled to reduce variation in the data about the fitted line.

For TI inactivation note that for a difference of 3.3 min between T* and the AI value for 100°C inactivation, a difference in PDIAI value of 9.5% occurred. Due to the faster rate of biologically active components inactivation and protein insolubility at 100°C, controlling blanching time would be critical to allow for both deleterious components inactivation and maximum protein solubility.

CONCLUSIONS

THE "LIMITING FACTOR" FOR BLANCHING of dehulled Williams was trypsin inhibitor, based on the AI value for TI being longer than for U or LO. The development of a first order fitted line of any thermal inactivation process allows for control of TI inactivation. It is not necessary to have data along the entire inactivation reaction rate line, but only at two sample treatments where confidence and prediction intervals have the widest range (or greatest uncertainty), namely at short and long processing times. Knowledge of the first order inactivation curve allows one to predict processing "target" critical inactivation times where the effects on various functional properties can be considered. If, as with protein insolubilization, additional functional properties have a first order response to processing, such values can also be quantitatively determined. Properties of the sample may be analyzed to optimize these results, as well as cause adequate biologically active components inactivation.

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Wheat Flour Protein Concentrate Characterization by Biochemical, Physicochemical and Baking Tests

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ABSTRACT

Protein concentrates before and after defatting from hard and soft wheat flours by water and 1% NaCl solution were characterized by lipid, sodium dodecyl sulphate (SDS) sedimentation, solubility, and breadmaking. Sedimentation values were higher for hard wheat flours than for soft, and in both about three times higher than in separated glutens. Solubility of proteins in SDS was reduced from ≈23 to 31% in flours to ≈3 to 19% in gluten concentrates. Defatting did not affect electrophoretic patterns but indicated lowered protein extractability. Gliadins and HMW glutenins were lower in extracts of separated glutens than in the extracts of doughs. Protein concentrates from fractionation with 1% salt solution, differed widely from those separated with water. This especially affected water absorption, dough development, and baking performance.

Key Words: wheat flour, gluten, breadmaking, loaf volume

INTRODUCTION

A SIMPLE FAST PROCESS of flour fractionation has been reported by Czuchajowska and Pomeranz (1993). It involves sequential combination of dough formation, followed by dough disintegration and centrifugation of the resulting batter. Yields of six fractions (prime starch, two gluten layers, tailings starch, insoluble fiber, and water solubles), their water and protein contents and recoveries were described. They determined the yield of a protein concentrate (gluten) and the effect of several factors on composition of two gluten layers. These factors included class of wheat, defatting, extraction with 1% salt solution, dough relaxation, and application of two levels of water for fractionation.

Our objective was to examine the quality and vitality of gluten from the above process. Several tests were used to characterize the two gluten layers (Fig. 1) from undefatted and defatted flours fractionated with 1% salt (NaCl) solution or water. Another objective was to determine how the two gluten layers differed. A third objective was to determine physical properties of dough and baking performance of gluten when it was used to increase protein content of a low-protein wheat flour.

MATERIALS & METHODS

WE EXAMINED 31 protein concentrates prepared on a laboratory scale. In addition we compared a commercial gluten and four flours. The flours were obtained from commercial hard wheat, commercial soft wheat, laboratory hard wheat, and laboratory soft wheat. Flour protein contents (Nx5.7, 14% moisture basis) were 12.6%, 8.9%, 9.5%, and 14.2%, respectively. The flours, concentrates and gluten were evaluted.

Lipid contents

Free lipids were determined by extracting samples with petroleum ether (PE) (AACC, 1983). Total lipids were extracted with water-saturated n-butanol. Two grams of gluten (dry basis) were extracted three times with 20 mL water-saturated n-butanol in a high speed blender (Ivan Sorvall, Inc., Newtown, CT). The solvent which contained extracted lipids and gluten particles was filtered after each extraction through a Büchner funnel fitted with a microfiber filter. Combined extracts were dried on a rotary evaporator under vacuum. Lipids were dissolved in a PE: ethanol (EtOH) mixture to assist in removal of water, evaporated under vacuum, dissolved in PE, centrifuged 20 min at 1,000 × *g*, transferred to preweighed vials, and dried by evaporation under nitrogen. The flours were also defatted with chloroform (prior to fractionation) as described by Czuchajowska and Pomeranz (1993).

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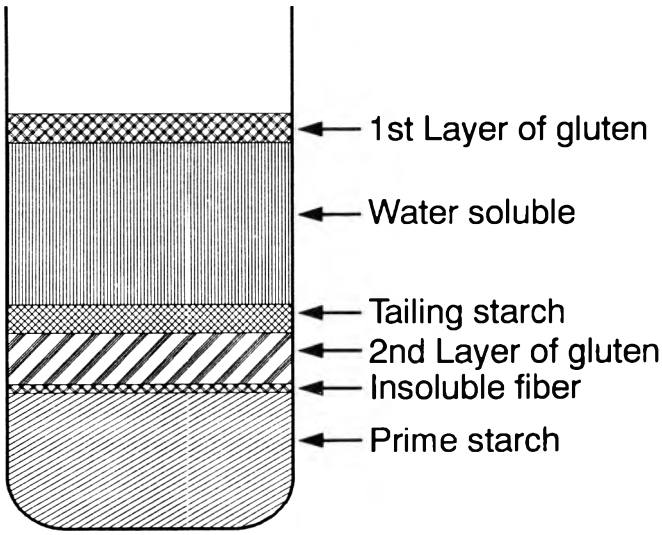


Fig. 1—Layers from fractionation.[*From Czuchajowska and Pomeranz, 1993.*]

Sodium dodecyl sulphate (SDS) sedimentation volume

The method of Axford et al. (1978) as modified by McDermott (1985) was used. Gluten samples containing 500 mg protein were tested. Samples were tested on a constant protein basis to provide meaningful comparisons of protein quality, independent of quantity.

Protein extractability

Samples were fractionated by extraction with 70% (v/v) EtOH and an aqueous solution of 1.5% (w/v) (SDS) into two protein fractions and a residue. A sample (1g, dry basis) was suspended in 4 mL of 70% (v/v) EtOH, vortexed at 10 min intervals for 1 hr and centrifuged at 2,440 × *g*. The supernatant was collected, the residue was suspended in 4 mL 70% (v/v) EtOH, vortexed again for 1 hr, and centrifuged 30 min at 2,440 × *g*. The supernatants were combined. The residue was resuspended in a 1.5% (w/v) SDS, vortexed at 10 min intervals for 1 hr, and centrifuged 20 min. EtOH-soluble extracts and SDS-soluble and SDS-insoluble extracts were freeze-dried. Protein content was determined by thermal conductivity using a Nitrogen Determination System FD-438 (LECO Co., St. Joseph, MI). Protein recoveries, calculated as the sum of protein contents in all fractions, ranged from 95.1 to 99.7% and averaged 97.3%.

Electrophoresis of gliadin

Samples (containing 50 mg protein) of the flour, of lyophilized dough mixed with water and salt, or of gluten fractions from the doughs were extracted with 200 µL 70% ethanol in 1.5-mL microcentrifuge tubes. The mixtures were vortexed every 10 min for 1 hr at 21°C (room tem-

Table 1—Characteristics of flour samples

Flour	Lipids (%) Free	Lipids (%) Total	SDS sedimentation volume (mL)	% of Gluten protein[a] Ethanol-soluble	% of Gluten protein[a] SDS-soluble	% of Gluten protein[a] SDS-insoluble
Laboratory hard	0.92	1.78	80.0	45.9	22.9	31.2
Commercial hard	1.31	1.97	77.5	45.5	25.5	29.0
Laboratory soft	0.92	1.62	67.5	49.4	30.7	19.9
Commercial soft	1.19	1.73	57.5	47.8	26.9	25.3

[a] Corrected for recovery.

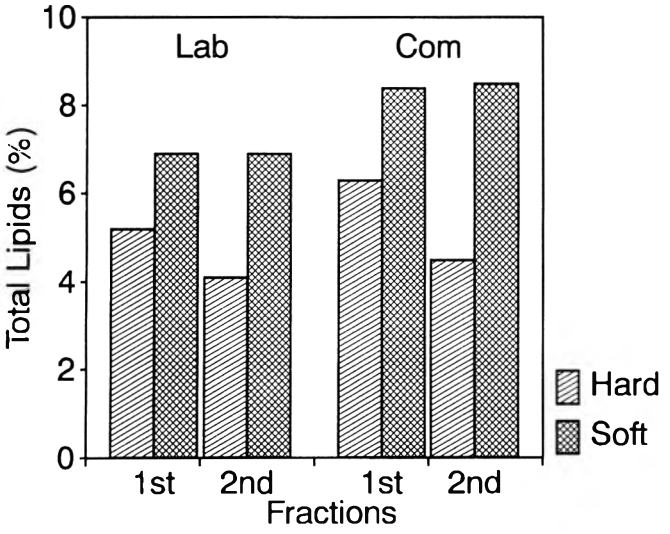


Fig. 2—Total lipids in the first and second gluten fractions separated from laboratory and commercially milled hard and soft wheat flours.

perature), and centrifuged for 10 min at 30,500 × *g* at 20°C. The supernatant was transferred to a new microcentrifuge tube and diluted to 200 µL with a solution containing 60% (v/v) glycerol, 0.125 (w/v) aluminum lactate in 70% (v/v) ethanol and 3% (w/v) methyl violet tracking dye. Gliadin extracts were stored at 4°C until further use. Each slot in the gel was loaded with 10 µL of solution. The ethanol extract from the wheat cultivar Marquis was used for comparison with the ethanol extract of tested samples. Gliadins were extracted twice from all samples and each extract was analyzed twice on separate gels. Polyacrylamide gel electrophoresis (PAGE) was based on the procedures of Khan et al. (1985) and Lookhart et al. (1982).

Electrophoresis of glutenin extracts

The residues from gliadin extraction in open microcentrifuge tubes were kept at 20°C 2–3 days and used to extract glutenin with an SDS buffer solution (without and with mercaptoethanol) and were separated by PAGE in the presence of SDS using the method of Ng and Bushuk (1987). The gels were scanned using a densitometer and photographed immediately through an orange filter (no. 02 from NPS Optical Filters, Japan).

Relative mobility of gliadin bands was standardized by comparison with mobility of protein bands from the cultivar Marquis in the same gel. For gliadins, the electrophoregram was divided into four regions according to the typical gliadin solubility classification: omega, gamma, beta and alpha; the total area was calculated as a sum of those regions. Results of gliadin and glutenin quantification were expressed as means of total peak areas. The reported total peaks were means of two determinations on two extracts from separate gels. Neither processing of flour into dough nor separation of dough into gluten fractions consistently affected reproducibility of electrophoretic patterns. Also reproducibility was not affected by type of the flour (hard or soft) source of flour (commercial or laboratory), defatting, or for gliadins and glutemns. The mean coefficient of variability was about 6% and apparently depended on the sum total of experimental erros from isolation of proteins, separation by electrophoresis, staining and destaining, and densitometric quantification.

Table 2—Quality of gluten as determined by the sodium dodecyl sulphate (SDS) sedimentation test

Extractant	Gluten layers	SDS sedimentation volume (mL) Hard wheat Laboratory	Hard wheat Commercial	Soft wheat Laboratory	Soft wheat Commercial
		Nondefatted flour			
Water	First	30.0[a]	28.0[a]	20.0[b]	21.0[c]
	Second	28.5[b]	27.0[ab]	22.0[a]	23.5[a]
1% NaCl	First	no layer	—	—	—
	Second	25.5[c]	26.5[b]	19.0[c]	23.5[a]
		Defatted flour			
Water	First	16.5[e]	26.5[b]	18.0[d]	18.0[e]
	Second	28.5[b]	23.5[c]	20.5[b]	22.0[b]
1% NaCl	First	15.0[f]	22.5[d]	19.0[c]	20.0[d]
	Second	24.0[d]	no layer	18.0[d]	20.0[d]

[a-f] Means with different superscripts in a column are significantly different at the 5% level.

Physical dough testing

Physical dough properties were evaluated by mixograph according to Finney and Shogren (1972). Dough from the soft wheat flour was fortified by protein concentrate (gluten) to increase protein level from 8.9 to 11.9%. Gluten samples were obtained from flours fractionated by water. They included the second gluten layer (protein concentrate) from two laboratory milled hard wheat flours, hard and soft commercial flours and a commerical gluten. In addition, gluten separated as 2 layers from a commercial hard wheat flour was evaluated. The two layers were studied separately. Mixograph development curves of doughs fortified by gluten fractions were the basis for estimation of optimum water absorption and mixing times.

Bread baking test

The low protein flour was fortified (on a replacement basis) by gluten to increase protein content from 8.9 to 11.9% (Czuchajowska and Pomeranz, 1990). Bread was baked with each gluten at a fixed water absorption (58%) and mixing time 2 min. The baking performance of the 31 protein concentrates from the four flours (each, defatted and nondefatted, fractionated by water and a 1% salt solution), was determined whenever 2 protein layers were obtained during fractionation. In addition to the fixed baking schedule, bread was baked also under optimized conditions of mixograph water absorption and mixing time. Protein concentrates (gluten) from nondefatted flour, 1st and 2nd layer from the commercial hard wheat flour fractionated by using a water:flour ratio of 2.5:1.0, were used for these tests. Amounts of 1st layer glutens separated from the other three flours plus cv Klasic (11.8% protein)(at a water: flour ratio of 2:0 to 1.0) were negligible and not evaluated by the optimized procedure. Varying separation conditions, such as water to flour ratio and eliminating relaxation time were used to minimize amount of total and waste water, shorten fractionation time, and provide a single (instead of double) protein concentrate layer. Those factors were described previously (Czuchajowska and Pomeranz, 1993) and were the subject of detailed evaluation. The straight dough baking procedure of Finney (1984), was used. Loaf volume was measured immediately after baking and results were reported as increase per 1% of added gluten protein. Bread crumb structure was evaluated (based on appearance) by an experienced laboratory baker 1 hr after baking on a 1 to 10 scale (10 = excellent). Texture was measured by a Fudoh Rheometer (Tokyo, Japan) fitted with a disc probe of 1 cm^2 and automatic stop adjusted to permit 1 cm penetration into a 4 cm thick bread section.

Statistical analysis

All tests were done at least in duplicate and were averaged. Statistical analyses were carried out using the *Statistical Analysis System* (SAS Institute, Inc., 1982).

RESULTS & DISCUSSION

Flour characteristics

Characteristics of the four flours were compared (Table 1). Commercially milled flours were higher in total lipids than laboratory milled flours. The hard wheat flours were higher in SDS sedimentation volume than the soft wheat flours and the labo-

Table 3—Protein solubility fractions of gluten [Soluble Protein(%)]

Extractant	Gluten layers	Hard wheat flour: Laboratory milled: Soluble in EtOH	Hard wheat flour: Laboratory milled: Soluble in SDS	Hard wheat flour: Laboratory milled: SDS Insoluble	Hard wheat flour: Commercially milled: Soluble in EtOH	Hard wheat flour: Commercially milled: Soluble in SDS	Hard wheat flour: Commercially milled: SDS Insoluble	Soft wheat flour: Laboratory milled: Soluble in EtOH	Soft wheat flour: Laboratory milled: Soluble in SDS	Soft wheat flour: Laboratory milled: SDS Insoluble	Soft wheat flour: Commercially milled: Soluble in EtOH	Soft wheat flour: Commercially milled: Soluble in SDS	Soft wheat flour: Commercially milled: SDS Insoluble
Nondefatted flours													
Water	First	38.1^{d}	3.2^{f}	53.8^{b}	37.6^{e}	3.9^{a}	54.2^{a}	37.0^{c}	7.5^{f}	51.5^{a}	39.5^{d}	6.9^{c}	51.0^{ab}
	Second	38.7^{d}	8.0^{c}	49.0^{c}	41.2^{c}	5.5^{cd}	52.1^{a}	40.2^{b}	8.6^{e}	46.0^{a}	39.1^{d}	5.4^{de}	52.1^{a}
1% NaCl	First	33.8^{e}	6.1^{d}	59.3^{a}	—	—	—	—	—	—	—	—	—
	Second	46.0^{b}	4.6^{e}	48.7^{c}	46.1^{a}	4.8^{de}	48.3^{b}	47.3^{a}	9.5^{d}	41.9^{b}	45.4^{a}	6.1^{cd}	47.2^{de}
Defatted flours													
Water	First	39.0^{d}	5.9^{d}	53.0^{b}	39.0^{d}	6.2^{bc}	53.5^{a}	36.9^{c}	10.4^{c}	51.6^{a}	38.8^{cd}	6.5^{c}	50.1^{bc}
	Second	42.8^{c}	13.6^{h}	41.0^{d}	39.6^{d}	17.6^{a}	37.3^{c}	40.2^{b}	16.9^{a}	42.7^{b}	39.0^{d}	13.5^{a}	46.9^{de}
1% NaCl	First	45.7^{b}	3.9^{ef}	49.9^{c}	43.8^{b}	7.1^{b}	47.7^{b}	41.9^{b}	6.6^{g}	48.8^{a}	42.5^{b}	4.6^{a}	48.8^{cd}
	Second	47.5^{a}	15.0^{a}	33.8^{e}	46.1^{a}	18.7^{a}	31.5^{d}	42.6^{b}	12.4^{b}	41.1^{b}	42.1^{bc}	11.8^{b}	45.8^{e}

[a-h] Means with different superscripts in the same column are significantly different at the 5% level.

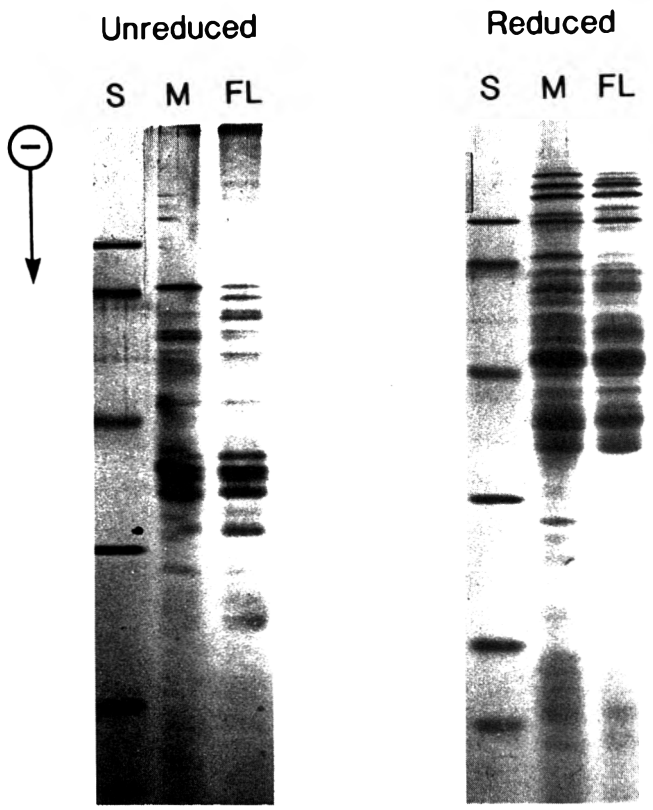


Fig. 3—Sodium dodecyl sulfate-polyacrylamide gel electrophoregrams of unreduced and reduced glutenin from nondefatted hard wheat flours. S = standard proteins (from top to bottom with decreasing molecular weight). M = Marquis. FL = laboratory milled hard wheat flour. (Composite picture).

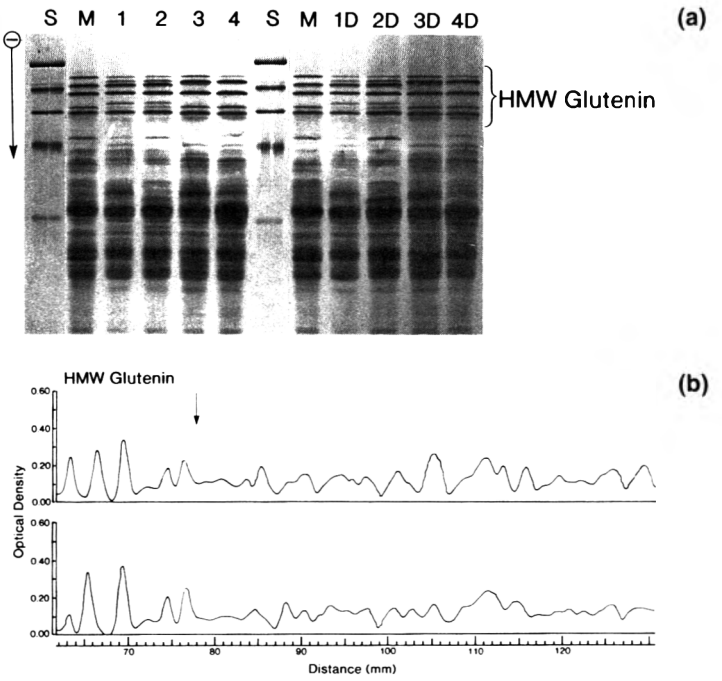


Fig. 4—(a) SDS-PAGE of reduced glutenin of hard and soft wheat flours, nondefatted (1, 2, 3 and 4) and defatted (1D, 2D, 3D and 4D). 1 and 1D = laboratory milled hard wheat; 2 and 2D = commercially milled hard wheat; 3 and 3D = commercially milled soft wheat; 4 and 4D = laboratory milled soft wheat. M = Marquis and S = standard proteins (from top to bottom with decreasing molecular weight). **(b) Densitometric tracings of the SDS-PAGE patterns of Marquis (upper) and of defatted laboratory soft wheat flour (lower).** Arrow indicates high molecular weight glutenins.

ratory milled flours were higher in SDS sedimentation than commercially milled flours. The hard wheat flours were higher in SDS insoluble proteins (primarily high molecular weight glutenins) and lower in EtOH soluble proteins (primarily gliadins) than soft wheat flours. Amounts of SDS soluble proteins (primarily low and intermediate molecular weight glutenins) varied.

Gluten lipids

Total lipids were lower in glutens washed from laboratory milled than from commercially milled flours (Fig. 2) and from hard wheat flours than from soft wheat flours. In glutens from hard wheat flours, the second (major) gluten fractions were lower in total lipids than the first (minor) gluten fractions. Lipid contents ranged from 4.1 to 6.3% from hard and 7.0 to 8.4% from soft wheat flours. These values confirmed the data reported for commercial glutens by Bushuk and Wadhawan (1988).

Sodium dodecyl sulphate sedimentation volume

One of the tests selected for measuring gluten quality was the SDS sedimentation volume. The use of the SDS sedimentation test in evaluating breadmaking quality of commercial wheat glutens has been described in several reports. In a study of McDermott (1985) in only 23 (out of 30) samples was there a positive correlation between SDS sedimentation and baking performance. In a study by Czuchajowska and Pomeranz (1990), SDS sedimentation volumes (on a 500 mg protein basis) were

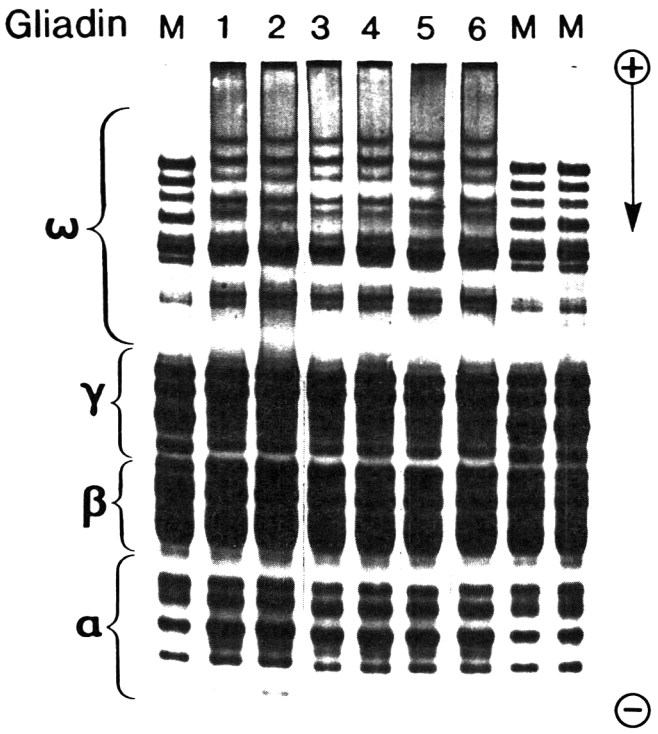


Fig. 5—Gliadin polyacrylamide gel electrophoregrams for nondefatted commercially milled hard wheat flour. 1 = flour; 2 and 5 = dough mixed with water and salt, respectively; 3 and 4 = first and second gluten, respectively, for dough mixed with water; 6 = second gluten from dough mixed with salt. M = flour of cv. Marquis used as reference. Migration was from top to bottom (+ to −). Greek letters refer to conventional electrophoretic mobility designations of gliadin groups.

Table 4—Effects of flour defatting on total gliadins, total HMW glutenins, and glutenin to gliadin ratios in various flours

Flour	Gliadin	HMW Glutenin	Glutenin/ Gliadin
Laboratory hard defatted	21.27[a]	2.91[a]	0.14[a]
	17.51[b]	2.81[a]	0.16[a]
Commercial hard defatted	23.03[a]	3.05[a]	0.14[a]
	17.36[a]	2.66[a]	0.16[a]
Laboratory soft defatted	26.22[a]	2.74[a]	0.10[b]
	20.71[a]	2.55[a]	0.13[a]
Commercial soft defatted	20.99[a]	3.17[a]	0.15[b]
	17.36[a]	3.01[a]	0.18[a]

[a-b] Mean values followed by different letters indicate statistical differences at the 5% level.

Table 5—Effects of processing nondefatted flour on total gliadins, total HMW glutenins, and glutenin to gliadin ratios in doughs and glutens from various flours

Test product	Gliadin	HMW Glutenins	Glutenin/ Gliadin
Laboratory hard			
Dough	20.92[a]	2.89[a]	0.14[a]
Gluten	17.47[a]	2.72[a]	0.16[a]
Commercial hard			
Dough	23.95[a]	2.67[a]	0.11[a]
Gluten	21.28[a]	2.43[a]	0.12[a]
Laboratory soft			
Dough	20.52[a]	2.83[a]	0.14[a]
Gluten	21.43[a]	2.31[a]	0.11[a]
Commercial soft			
Dough	26.52[a]	2.58[a]	0.10[a]
Gluten	25.97[a]	2.03[b]	0.08[b]

[a-b] Mean values followed by different letters indicate statistical differences at the 5% level.

Table 6—Increase in loaf volume of bread per 1% per cent of added gluten protein [Loaf volume increase (cm^3)]

Treatment	Gluten layers	Hard wheat flour: Laboratory milled	Hard wheat flour: Commercially milled	Soft wheat flour: Laboratory milled	Soft wheat flour: Commercially milled
		Nondefatted flour			
Water	First	48.3[ab]	45.4[a]	35.4[a]	no sample
	Second	30.8[d]	23.8[b]	22.9[bc]	32.5[a]
Salt	First	50.0[a]	—	—	—
	Second	40.0[c]	42.1[a]	28.8[ab]	22.5[b]
		Defatted flour			
Water	First	23.4[e]	19.1[bc]	19.6[c]	10.8[c]
	Second	15.0[f]	0.0[d]	2.1[d]	0.0[d]
Salt	First	43.4[bc]	16.3[c]	24.9[bc]	15.9[bc]
	Second	16.7[f]	−22.9[e]	5.4[d]	0.0[d]

[a-e] Means with different superscripts are significantly different at the 5% level.

≈25 to 30. In our results (Table 2), the sedimentation values of the protein (gluten) concentrates were only a fraction (about 1/3) of those of the flours (Table 1). The SDS volumes of glutens from hard wheat, nondefatted flours ranged from 25.5 to 30.0 mL. Glutens from soft wheat, nondefatted flours had lower SDS volumes (19.0 to 23.5 mL) than those from hard wheat flours. Fractionation in 1% NaCl solutions, generally, decreased SDS values.

SDS volumes of glutens from defatted hard wheat flours, generally, were lower (16.5 to 28.5 mL) than those of glutens from nondefatted flours. The pattern for glutens from soft wheat flours was similar to that of hard wheat flours. Results indicated that defatting flours affected the aggregation and probably breadmaking potential of gluten proteins. Note that defatting increased the solubility of proteins in the SDS solvent.

Protein extractability

Protein extractability from the 31 separated protein concentrates (gluten fractions) was compared. Extractability in SDS was reduced from about 23 to 31% in flours (Table 1) to 3 to 19% in gluten concentrates (Table 3). The amount of proteins insoluble in SDS increased from about 20 to 31% in the flours to 34 to 59% in gluten concentrates. Amounts of ethanol soluble proteins changed relatively little. Defatting the flours increased (with two exceptions) the amounts of SDS soluble proteins. The second (major) gluten fraction was higher (with two exceptions) in SDS soluble proteins and lower in SDS insoluble proteins than the first (minor) gluten fraction.

PAGE and SDS electrophoresis

The electrophoretic patterns of unreduced and reduced SDS-protein extracts from residue fractions were compared after extraction of laboratory milled hard wheat flour and Marquis with 70% (v/v) ethanol (Fig. 3). The SDS-PAGE patterns of samples investigated under unreduced conditions showed traces of low mobility bands.

SDS-PAGE results of unreduced glutenin preparations reported by Bushuk et al. (1980), Khan and Bushuk (1979) and Simmonds (1980) suggested two groups of proteins in the glutenin complex: those that entered the SDS-gel and those that remained at the point of sample application. As shown (Fig. 3), the glutenin (high molecular weight) proteins had either not been extracted or (if present) largely remained in the starting slots of the gel. Singh et al. (1987) found that "triplet proteins" (a set of three bands with very low mobility) occurred after SDS-PAGE of unreduced total protein extracts. Similar results were presented by Sievert et al. (1991). However, in the presence of reducing agents such as 2-mercaptoethanol in SDS-extracts, the glutenin could be released from residue as polypeptide

Commercial Soft

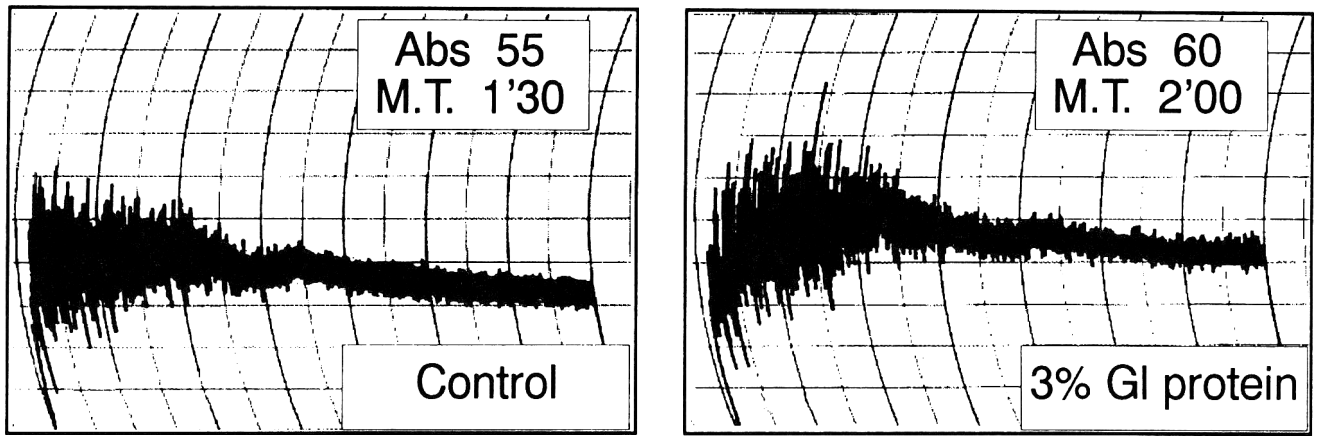


Fig. 6—Mixographs of soft wheat flour; left control, right enriched by 3% protein from cv Klasic wheat flour.

Table 7—Proof height, loaf volume, and loaf volume increase of bread baked under optimized conditions

Sources of gluten	Proof height (cm)	Loaf volume (cm^3)	Loaf vol. increase/ 1% gluten proteins
Control - no gluten (commercial soft)	6.65[d]	605[e]	—
Commercial hard 1st layer (2.5:1)[f]	7.10[abc]	745[c]	46.7[bc]
Commercial hard 2nd layer (2.5:1)[f]	7.07[bc]	715[d]	36.7[d]
Commercial soft (2:1)[f]	7.00[c]	722[d]	38.9[d]
Commercial hard (2:1)[f]	7.10[abc]	748[bc]	47.8[bc]
Laboratory hard - HWS (Klasic 2:1)[f]	7.23[ab]	813[a]	69.4[a]
Laboratory hard (2:1)[f]	7.03[bc]	757[b]	50.5[b]
Commercial gluten	7.03[a]	760[b]	58.7[b]
LSD	0.202	28.40	9.56

[a-e] Mean values followed by different letters indicate significant differences at the 5% level.

[f] Water-to-flour ratio used in fractionation.

"subunits." These may be fractionated by SDS-PAGE on the basis of molecular size alone.

The quantification of protein separated by SDS-PAGE was carried out only with reference to reduced glutenin (high molecular weight - HMW). HMW glutenins were classified on the basis of 5 subunits using the cultivar Marquis and high or low molecular weight SDS-PAGE standard as references (Fig. 4). Densitograms of reduced glutenins from Marquis flour and defatted laboratory milled soft wheat flour were also compared (Fig. 4a).

The flours differed in electrophoretic patterns, reflecting genetic variability (Fig. 4). Defatting did not affect qualitative patterns. Defatting reduced, however, the intensity of separated bands, probably a result of lowered extractability of proteins as stated by Chung and Pomeranz (1978). The effect of defatting on intensity of electrophoretic bands was more pronounced for HMW glutenins from hard than from soft wheat flour (Fig. 4).

The qualitative and quantitative PAGE patterns of gliadins were retained by processing of the nondefatted flour into dough and gluten fractions (Fig. 5). While defatting consistently reduced the amounts (intensity) of gliadins from all flours, an effect on the qualitative pattern was recorded for gliadin proteins isolated from the commercial hard wheat flour, only (data not shown). Defatting that flour, resulted in an additional separation of the fast migrating ω-gliadins. The reason for that effect on the ω-gliadins which may have resulted from some treatment during production of the commercial flour.

Neither defatting of flours nor processing into dough or gluten fractions affected patterns of HMW glutenins (data not shown). As indicated, defatting lowered both the amounts of gliadins and HMW glutenins in the 4 flour extracts (Table 4). There was also, as a result of defatting, an increase in calculated glutenin: gliadin ratios. Similarly, both the amounts of gliadins and HMW glutenins were lower in extracts of separated glutens than in extracts of doughs (Table 5). However, lowering amounts of gliadins and HMW glutenins resulted in no consistent differences in calculated glutenin:gliadin ratios.

Our results demonstrate the limited effect of physical treatments on patterns of gluten proteins. Those proteins undergo rather harsh treatment during electrophoresis. Relatively minor chemical changes (as they relate to functional properties) took place during dough mixing and centrifugation. Defatting resulted in more pronounced changes than extraction with salt solution.

The absence of major modifications in our fractionation procedure was critical to retention of end use properties of gluten proteins. Fortuitously, extraction of non-defated flour with water produced the best gluten and could be used, under appropriate conditions, to minimize the amount of total water and waste water in production of a well separated, single, functional gluten layer (Czuchajowska and Pomeranz, 1993).

Bread baking

Effects of adding concentrates (replacement basis) to increase flour protein from 8.9 to 11.9% were compared on loaf volume increase (per 1% of added protein) when baked under fixed conditions (Table 6). Note that defatting changed the relative amounts of the two gluten fractions, their protein contents, and protein composition, as indicated by differences in SDS sedimentation tests (Table 2) and protein solubilities (Table 3).

Gluten samples from nondefatted flours substantially increased loaf volume and improved bread crumb and texture. As a result of enrichment by gluten from nondefatted flours, bread scores were improved. Results of texture determination, generally, correlated with effects on loaf volume; they were improved by adding gluten except for toughening of bread crumbs as a result of adding gluten from defatted flours (results not shown). The increase in volume ranged from 22.5 to 50.0 cm^3/1% added gluten protein. Data indicate that the protein concentrates contained vital gluten and performed well in baking. The effects were comparable to those on breadmaking of commercial glutens of high quality (Czuchajowska and Pomeranz, 1991). In

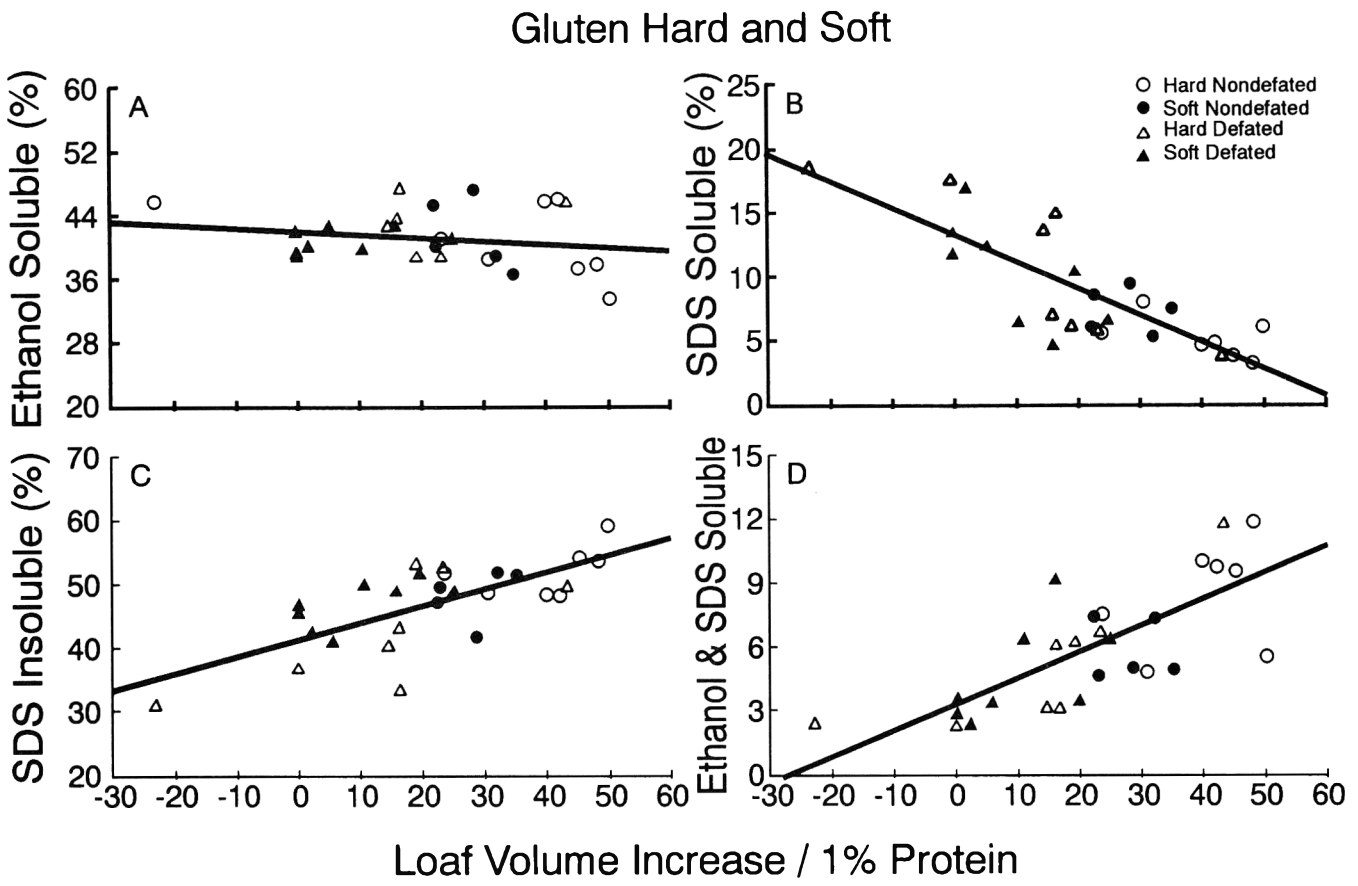


Fig. 7—Linear regression between loaf volume and ethanol extractable proteins, SDS extractable proteins, SDS inextractable proteins and ratio between ethanol soluble: SDS soluble proteins for glutens from nondefatted flours (from hard ○ and soft ▲ wheats) and defatted flours (△ from hard and ▲ from soft wheats).

Table 8—Statistical relationship between loaf volume increase (X) and SDS Sedimentation volume (Y) of glutens

Sample description	No. of samples	Slope	Intercept	Linear correlation coefficient
All samples				
Nondefatted	11	0.204	18.00	0.516
Defatted	15	−0.103	22.26	−0.327
Combined	26	0.092	20.47	0.321
Without one sample[a]				
Nondefatted	11	0.204	18.00	0.516
Defatted	14	−0.018	21.43	−0.046
Combined	25	0.135	19.92	0.480

[a] See text.

that study of 61 commercial glutens, the best glutens increased loaf volume from 26 to 50 cm^3/1% gluten protein.

Protein (gluten) concentrates from the strong, hard wheat flours were more effective than those from concentrates from the weaker, soft wheat flours. The minor (first) protein fraction was more effective than the major (second) fraction. No consistent effect of extracting the proteins in a salt solution (as compared to water) was observed. Defatting resulted in less effective gluten concentrates. That effect may have resulted from one or more of three causes. Important, useful wheat flour components (i.e. polar lipids), may have been removed. A shift-change in protein solubility or some modification of gluten proteins to make them less effective may have occurred.

The physical properties of fortified doughs were improved by addition of gluten separated from both the hard and soft wheat flours. Fortification increased water absorption and stabilized mixing properties (Fig. 6).

Results of bread baking under optimized conditions were compared (Table 7). Bread baked with the 1st and 2nd gluten layers from commercial hard wheat flour fractionated by water differed less (10 cm^3) than bread baked under fixed conditions (22 cm^3, see Table 6). Gluten from the 1st layer greatly increased loaf volume when baked under fixed and optimized conditions, (45.4 cm^3 and 46.7 cm^3/1% of gluten proteins, respectively). Gluten from the second layer performed better when baked under optimized conditions (increase of 36.7 cm^3/1% protein) than when baked under fixed conditions (increase of 23.8 cm^3/1% protein).

Results indicate that gluten from the first layer probably interacted readily with endogenous gluten of soft wheat flour and performed well even under fixed conditions. The interaction between exo- and endogenous glutens appeared important for gluten's general performance, as reported by Czuchajowska and Pomeranz (1993).

When protein concentrates from the flours and commercial gluten samples were incorporated into soft wheat flour and baked under optimized condition, enrichment resulted in large increases in loaf volume (38.9 up to 69.4 cm^3/1% of gluten proteins, Table 7). Differences in performance of the glutens were significant and related to the flours from which the glutens had been isolated.

Relation between quality tests and breadmaking

To evaluate the baking performance of glutens from a given flour, but originating from the two layers of protein concentrates, gluten properties were correlated with baking performance under set conditions. Solubility of protein in SDS and extractability of proteins by water and 1% salt solution were compared from undefatted and defatted flours (Fig. 7).

The possible ineffectiveness from defatting was indicated by the better performance of the first minor (lipid rich) than of the second (major) gluten fraction (Table 6). Relationship was found between loaf volume increase from adding glutens and

Table 9—Probabilities and linear correlation coefficients between loaf volume increase per 1 per cent gluten protein and gluten parameter

Gluten parameter	Hard wheat flour				Soft wheat flour			
	Laboratory milled		Commercially milled		Laboratory milled		Commercially milled	
	Correlation	Probability	Correlation	Probability	Correlation	Probability	Correlation	Probability
Ethanol-soluble (%)	−0.426	0.292	−0.319	0.486	−0.04	0.932	0.132	0.083
SDS-soluble (%)	−0.84	0.009	−0.913	0.004	−0.876	0.01	−0.825	0.043
SDS-insoluble (%)	0.786	0.021	0.869	0.011	0.6	0.154	0.747	0.088
Ratio EtOH soluble: SDS soluble	0.732	0.039	0.951	0.001	0.788	0.035	0.763	0.078

the SDS sedimentation tests of those glutens (Table 8). Results were compared for all samples and also calculated after exclusion of one of the samples (first-minor fraction from hard wheat flour, laboratory milled, defatted, salt extracted; Table 6) which gave inconsistent results. Only the combined samples, after exclusion of the latter, showed a significant correlation ($p \leq 0.05$). Results confirmed the limited effectiveness of SDS sedimentation to predict breadmaking potential of commercially or laboratory produced glutens.

Gluten samples from defatted flours had 15% lower SDS volumes than those from nondefatted flours (Table 2). However, much larger differences were obtained in the complex, multi-step, multi-component baking test (Table 8), than in the relatively simple SDS test. Incorporation of glutens from defatted flours into dough may be much less reliable than incorporation of gluten from nondefatted flours. Glutens from defatted flours were much tougher and shorter than those from nondefatted flours.

The effects of flour defatting on gluten properties were in confirmation of the findings of Hoseney et al. (1970) who postulated that lipids were essential in gluten performance. The gliadin-lipid-gluten complex is required to form a "membrane" for satisfactory gas retention during bread baking.

The possible causes and effects between shift, variation, or ratio in protein solubility groups and performance in breadmaking were examined for glutens separated from the four flours (Table 9). No consistent or significant relationship between loaf volume increase and amount of EtOH soluble proteins was established. Whereas the amount of SDS-soluble proteins correlated negatively, the amount of SDS-insoluble proteins correlated positively, with loaf volume increase. Another high linear correlation occurred between loaf volume increase and ratio of EtOH-soluble to SDS-soluble proteins.

The relationship between loaf volume increase and solubilities of proteins in EtOH and SDS (and their ratios) for all samples were compared (Fig. 7). Combining all samples did not change the general patterns for the 4 separate groups. The statistical significance of the linear correlations for the combined samples increased considerably. The results are best considered with regard to loaf volume increases (of bread baked under fixed conditions) above and below 20 $cm^3/1\%$ added protein. That was the general dividing line between glutens from nondefatted and defatted flours. The overall correlation coefficient for EtOH soluble proteins was −0.207 (nonsignificant) and the linear regression line had a very small slope (−0.04). For the SDS soluble proteins, the correlation coefficient was −0.810 ($p < 0.0001$) and the slope was −0.210. For the SDS insoluble proteins, the correlation coefficient was 0.708 (0.0001) and the slope was 0.260. Finally, for the EtOH soluble:SDS soluble ratio the correlation coefficient was 0.740 (0.0001) and the slope 0.120. For wheat flours the balance between monomeric and polymeric proteins is essential for quality performance of gluten (Bushuk and Wadhawan 1989). Ineffective breadmaking wheat cultivars contain more gliadin and less residue proteins (SDS insoluble) than effective bread making cultivars (Orth and Bushuk, 1972).

CONCLUSIONS

PROTEIN CONCENTRATES from undefatted flours were vital, performed well in baking, and were comparable or more effective, on an equal protein supplementation basis, to high quality commercial glutens. Defatting with chloroform impaired end-use properties of protein concentrates. The SDS sedimentation test (on a constant protein basis) did not provide a single predictor of gluten quality for experimentally produced concentrates. Variability in breadmaking performance of protein concentrates could be accounted for by differences in extractability in SDS, non extractability in SDS, or ratio of extractability in EtOH to extractability in SDS, but not by extractability in EtOH alone. Variability among protein concentrates isolated from various flours was found in bread baked both under fixed and under optimized conditions.

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Near Infrared Analysis Potential for Grading Raisin Quality and Moisture

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ABSTRACT

Near infrared transmittance (NIT) spectroscopy was examined as a means for measuring raisin quality. High positive correlation was found between measured values and those predicted by NIT for bulk density, visual grade, and moisture content. NIT measures properties at the molecular level. Thus, results showed a relationship between raisin quality and some chemical constituents. NIT analysis has the potential for measuring general raisin quality as well as specific constituents.

Key Words: bulk density, moisture content, raisins

INTRODUCTION

SUN-DRIED RAISINS are graded for quality and moisture when they are delivered at processing plants. Grading may be by visual grading (VG) or by air flotation separation, called Airstream (AS) grading (USDA, 1989). Moisture content is measured using the Dried Fruit Moisture Tester (DFMT), an electrical conductivity measuring device calibrated to vacuum oven moisture measurement (AOAC, 1980).

Quality grading may be referred to as "maturity" grading, because it relates to maturity of grapes at the time they were harvested. Four visual grades have been described. In descending order they are A, B, C, and Substandard. Substandard raisins are "... practically lacking in flesh, with deep wrinkles and reddish in color..." C grade raisins are "... fairly well-matured, thin fleshed, deep wrinkles, thin ridges, reddish-brown in color." The remaining raisins are termed "B or Better" and combine the A and B grades. The B or Better fraction is described as "well-matured or reasonably well-matured, ... full-fleshed and round in appearance and may have fine wrinkles or shallow wrinkles with thick-edged ridges..." (USDA, 1989).

In an early attempt to standardize raisin quality, Chace and Church (1927) proposed use of bulk density. Better quality raisins had higher bulk density. Based upon an in-depth study over many years, Jacob (1942) described relationships between grape maturity and raisin quality. The weight of individual berries and bulk density increased with average maturity, total acidity varied inversely with maturity, and water insoluble solids varied inversely with maturity. Total sugar did not correlate well with maturity.

Fisher et al. (1958) developed the AS grader to measure the level of substandard raisins in an incoming crop. Air-flotation accurately measured the level of substandard raisins, and was efficient when compared to visual grading. The AS grader was subsequently applied to grading of grade C and B or Better fractions. Visual grading is used to calibrate the AS grader for determination of Substandard, C, and the B or Better fractions. The AS grader is used to grade most incoming raisins, but is seldom used to grade processed raisins, which are graded visually.

King and Stafford (1967), Stafford et al. (1968), and Stafford and Ponting (1969) examined alternate methods for establishing raisin quality grades, with emphasis on bulk density. They found correlation coefficients (r) of 0.819–0.836, between bulk density and AS grades, when moisture, temperature, container dimensions, and filling conditions were controlled. They also confirmed earlier findings of Jacob, that total acidity had a high negative correlation with maturity, but that there was low correlation between total sugars and the B or Better fraction. They concluded a grading system based upon chemical analyses, (such as acidity) may be useful but would be too costly to practice.

Our objective was to determine the potential use of an instrumental method for grading incoming raisins. Such a grading system could provide rapid analysis of raisins which could be correlated to visual grade. Such grading method could also be used to grade processed raisins, so that incoming raisins and processed raisins would be graded similarly. The AS grader accurately grades for substandard raisins. Thus, our objective was limited to determination of the C and the B or Better quality fractions and moisture content.

Near-Infrared Transmittance (NIT) spectroscopy was our instrumental method of choice. The absorption of near-infrared radiation and the application of such data to analyze a wide range of food and feed components have been described (Williams and Norris, 1987; Osbourne and Fearn, 1986). Birth et al. (1985) measured dry matter in onions using NIT spectroscopy, and Dull et al. (1989) demonstrated that NIT could evaluate soluble solids in cantaloupe. Moisture in dried dates was predicted using NIT (Dull et al., 1991).

Shenk et al. (1992) reviewed the application of NIT to agricultural products. They described the interaction of near-infrared radiation with solid particles in agricultural materials. Specular reflectance, diffuse reflectance, absorption, transmittance, refraction, and scattering occur and influence the shape of the spectra.

MATERIALS & METHODS

Sample materials

Raisins (100 lots) were selected from the 1991 crop, and a 10 kg sample was taken from each lot. The lots were selected to provide uniform distribution of quality, ranging from ≈40% to ≈90% grade "B or Better," as measured by AS sorter. Each sample was prepared in accordance with standard grading methods by official USDA graders and passed through an AS grader to remove substandard raisins. Remaining grade "C" and "B or Better" composite samples were retained for further analyses. All raisins were from Thompson Seedless grapes (which account for about 98% of commercial production).

Bulk density

A stainless steel beaker was used with nominal dimensions 204 mm dia and 250 mm ht with sidewall to bottom radius ≈10 mm, which contained 7.93 kg distilled water when filled to the brim (so top of meniscus was touching a straightedge across the top). This was filled with raisins and weighed to determine bulk density. To ensure uniform filling, the beaker was placed on a standard raisin-sizer shaker, which imparted a lateral motion to the container (amplitude 50 mm at 216 cycles/min). Raisins were added to the container by hand at a uniform rate, and a wooden straightedge was used to remove raisins above the brim. Weight was measured to the nearest 0.1g with an electronic balance. (Net weights of filled containers ranged from 4.913 to 6.056 kg.) The bulk temperature of samples was monitored at the time bulk density was measured, with a calibrated laboratory dial thermometer, to ensure sample temperature did not affect results. Conditions for measuring bulk density were similar to those recommended by Stafford et al. (1969).

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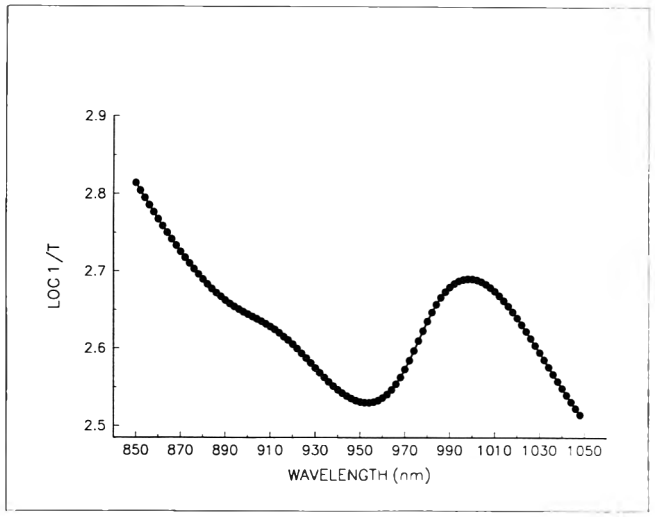


Fig. 1—Average spectrum of 92 lots of raisins.

Three replicate density measurements of each lot varied by <1%, and the average density was assumed to be the density of the lot. All density measurements were made within 5 mo of harvest.

NIT scans

An Infratec 1255 Food and Feed Analyzer (Infratec 1255) (Perstorp Analytical Sales, Silver Spring, MD) was used to collect NIT spectra. This is an NIT spectrophotometer, which operates in the region of 850 nm to 1048 nm, and measures absorbance at 2 nm increments. Transmittance mode was used because it requires little sample preparation, which is potentially important for in-field grading systems. The sample container was a cylindrical cup, 110 mm diam and 25 mm deep, with volume 240 mL. The bottom of the sample cup contained five equally spaced windows of about 20 mm diam, located about midway between the center and wall of the cup. The incoming beam was about 10 mm in diameter. The detector was about 10 mm square, and was located about 10 mm below the center of the window. The sample cup rotated between scans, so that spectra were taken at five nonoverlapping locations over the surface of the sample. For our tests, two scans were made at five locations, and the 10 spectra were averaged to one spectrum and stored on floppy disks.

Twenty subsamples (240 mL each) were taken from each raisin lot and placed in glass jars, numbered 1 to 20. Each subsample was scanned resulting in 2000 stored spectra for 100 samples. All scans were completed within 7 mo of harvest.

Visual grades

From the 20 subsamples, five were randomly selected for visual analysis of grade. A panel of three highly-experienced, trained graders simultaneously graded each sample and arrived at a consensus grade. Prior experience of the United States Department of Agriculture, Agricultural Marketing Service (USDA/AMS) indicated subjective visual grading results were more consistent and reproducible when graders simultaneously graded samples and arrived at a consensus, than when individual graders separately analyzed samples and averaged results. The panel weighed each subsample, removed C grade raisins, and recorded weights of C and remaining B or Better fractions. The ratio of B or Better fraction to total sample weight × 100 was the visual grade value, recorded as % B or Better. The average of visual grades for the five subsamples was assumed to be the visual grade for the lot.

The first eight lots graded by the panel were used to train graders to function as a panel and stabilize results. All subsequent evaluations were made on the remaining 92 lots. To extend the range for grade, the AS sorter was used to make five samples of nearly all C grade material and five samples of nearly all high grade B or Better raisins. Low grade raisins were all visually graded 5% B or Better, and high grade raisins ranged from 90% to 100% B or Better. All visual grading was complete within 7 mo of harvest.

Moisture

A set of samples from the 1989 raisin crop was evaluated to determine expected variations in moisture and chemical composition. The sample set comprised raisins from randomly selected lots with varying maturity grades, and no effort was made to select samples spanning specific moisture ranges. Samples were scanned within 9 mo of harvest, using techniques described for scanning grade samples. Raisins were analyzed for moisture by the vacuum oven method for moisture analysis of raisins (AOAC, 1980). A random selection of raisin lots from the 1991 crop that had been analyzed for visual grade was also evaluated for moisture. Samples from the 1991 crop were analyzed using the same vacuum oven and preparation as used for 1989 samples. After scanning, samples to be analyzed for moisture were held in sealed glass jars. Vacuum oven moisture analyses were completed within 10 mo of harvest.

Calibration

The Infratec 1255 was supplied with computer calibration software, Infratec Calibration Maker (ICM), Perstorp Analytical Sales, Inc. (1989), to be used for calibration models to predict "chemical" constituents from NIT spectra. For our tests, visual grade, moisture, and bulk density were treated as "chemical" constituents.

The ICM contains Principal Component Analysis (PCA) and Partial Least Squares (PLS) modules. PCA and PLS calibrations applied to NIT data have been described by Martens and Naes (1987). Haaland and Thomas (1988) further discussed PLS as a method of spectral analysis and related it to other quantitative calibration methods. PCA and PLS are full spectrum, data reduction, calibration methods that may utilize absorbance data from each measured wavelength of the spectrum. This contrasts with other calibration methods that rely on information from relatively fewer wavelengths. Single components, such as moisture, may be predicted based upon spectral values for a few wavelengths. However, visual grade and bulk density are probably influenced by relative levels of many constituents. These include acids, sugars, pectin, cellulose, moisture, proteins, and others. We used full spectrum methods to provide the greatest potential for accurate prediction.

Because PCA was available and recommended for calibration samples, we used it to select a subset of scans with the highest variation in principal components in spectral data. Corresponding chemical values for each selected scan were added, and PLS was used to make the calibration.

Two models were made for NIT prediction of visual grade (Model 1 and Model 2). For Model 1, PCA selected a set of 190 scans from 460 samples corresponding to five samples visually graded for each of the 92 lots. Twenty-five additional scans from the low grade samples and 25 scans from the high grade samples were added. Thus the calibration set contained a total of 240 scans.

To determine the best possible correlation between NIT scans and visual grades, Model 2 was made. For this 20 scans for each lot were averaged, the visual data from five subsamples were averaged, and calibration was made with PLS, using all 92 averaged scans and averaged visual data. No scans from the constructed high grade and low grade samples were included. The same 92 averaged scans were used to make an NIT calibration of bulk density by PLS. A moisture prediction model was made using 76 samples from the 1989 crop and 34 samples from the 1991 crop. Statistical evaluations were made using SAS (SAS Institute, Inc., 1987) and AXUM (Trimetrix, Inc., 1993).

Near-Infrared analysis software, NIRS 2 (Infrasoft International (ISI), Perstorp Analytical, Silver Spring, MD), was used to investigate spectral properties, including average spectra, first derivative spectra, standard deviations in the spectra, and correlation between spectral and chemical values.

RESULTS

THE AVERAGE NIT SPECTRUM for the entire set of scans was plotted as the logarithm of the inverse of the transmittance, log 1/T vs wavelength (Fig. 1). The spectrum was very smooth, as is characteristic of NIT in this region. Sharp peaks were not apparent. The first derivative of the average spectrum and the standard deviation of the first derivative were also compared (Fig. 2). This indicates bands where transmittance was affected and regions of spectra where variation occurred in the first derivative. The correlation was determined between the first derivative of the spectral values and visual grade, bulk density, and moisture (Fig. 3). Moisture showed very high correlation with several relatively narrow bands, indicating the excellent potential of NIT to determine moisture. Bulk density and visual

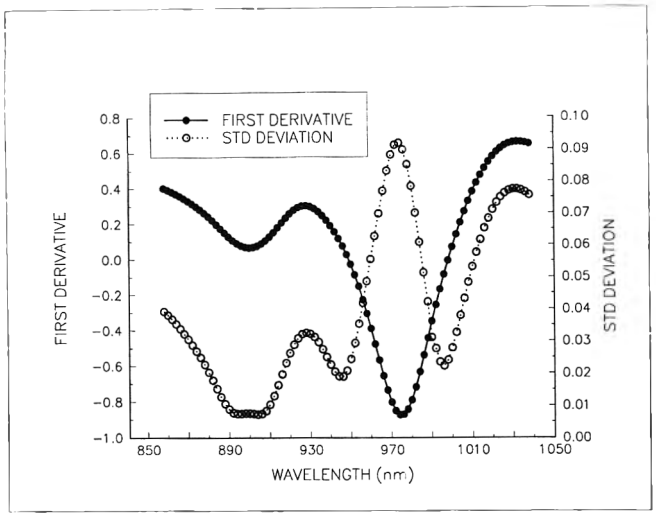


Fig. 2—First derivative of average near infrared spectrum and standard deviation of the first derivative as related to wavelength for 92 lots of raisins.

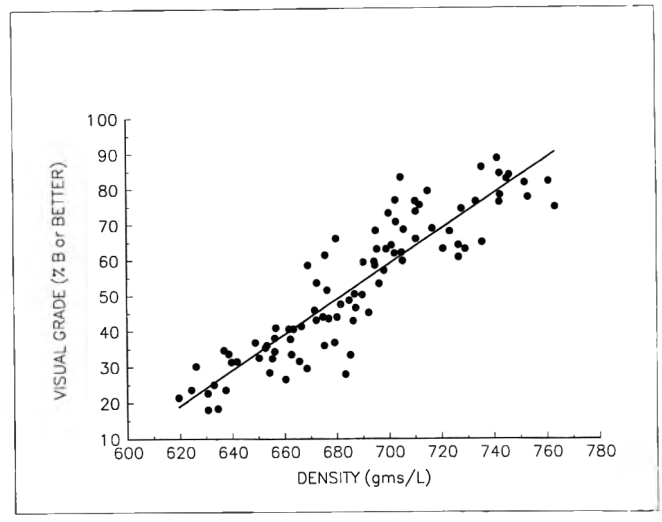


Fig. 4—Relationship between Visual Grade of raisins and bulk density (r = 0.91).

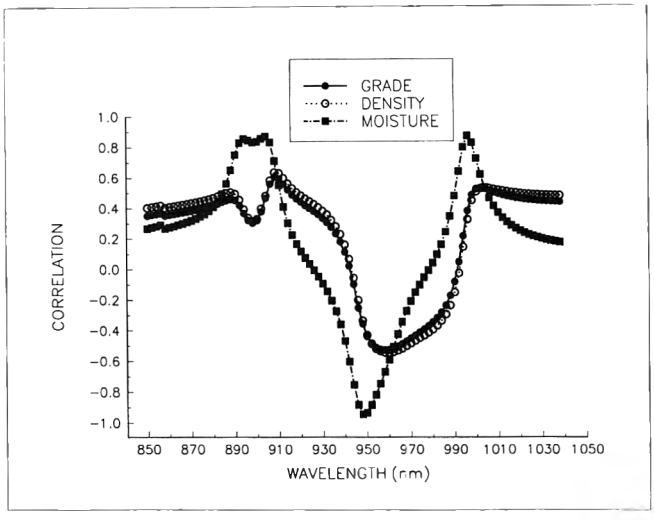


Fig. 3—Correlation between first derivative of spectra and measured grade, bulk density, and moisture values as related to wavelength.

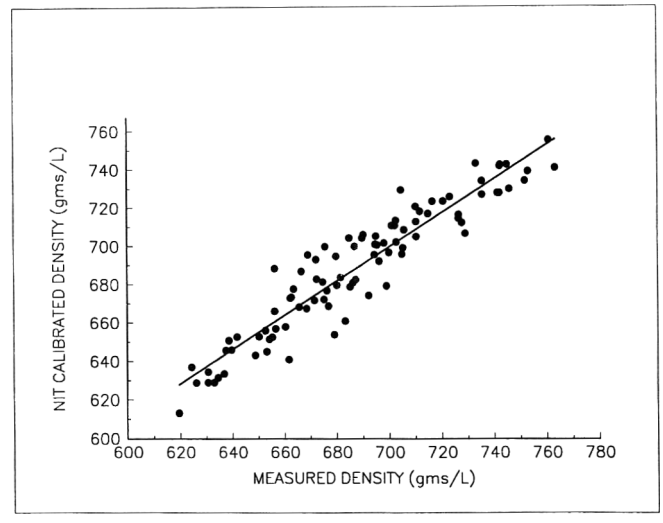


Fig. 5—NIT calibrated bulk density of raisins compared with measured bulk density (r = 0.94, RMSE = 12 gm/L).

grade as expected, showed lesser correlations throughout the spectrum.

Bulk density and visual grade values were highly interrelated (Fig. 3) in the spectral data. The correlations between visual grade and first derivatives were practically identical with the correlations of bulk density and first derivatives. Bulk density has been shown to correlate well with visual grade in other grading methods.

Bulk density

Bulk density was evaluated as a variable because it can be objectively measured, and it has shown good correlations with visual grade. The average visual grade for the 92 lots was compared with bulk density (Fig. 4). As expected a high correlation occurred (r = 0.91) between visual grade and measured density. The NIT calibration of bulk density (Fig. 5) correlation coefficient with measured values was quite high (r = 0.94).

Precision of the NIT calibration may be calculated by the Root Mean Square Error (RMSE).

$$RMSE = [\Sigma (Y_{ref.} - Y_{pred.})^2/df]^{1/2}$$

where $Y_{ref.}$ = reference, or laboratory, values of variable, $Y_{pred.}$ = NIT predicted values of variable, and df = degrees of freedom, n − 2, for linear regression, where n is the number of observations. The RMSE for the NIT calibration of bulk density was 12 g/L. Results indicate that NIT had good potential for predicting raisin bulk density.

Visual grade

The grade prediction Model 1 was used to predict grades for 20 scans from each of the 92 lots. Variance within lots was 32.91, and the variance between lots was 279.58 for NIT predicted values. For VG samples the variance within lots was 29.56 and the variance between lots was 388.05. The higher ratio of variance between lots to that within lots for VG shows that the NIT did not distinguish between grades as well as VG. The Coefficient of Variation (CV) for NIT predicted values was 10.70, and the CV for VG was 9.91, indicating that CV values for VG were only slightly lower than the CV for NIT prediction.

The NIT predicted results for each lot were averaged and compared with average visual grades (Fig. 6). Correlations between NIT predicted values and VG values was r = 0.94, and the RMSE = 6.24. Scans for each lot were also averaged to a single scan prior to prediction (Fig. 6). For this prediction r = 0.93, and RMSE = 6.23. The averages of predictions of the scans and predictions of the average of the scans coincided very closely.

A calibration model was also made from the 92 averaged scans. This model had a r = 0.96 and an RMSE = 6.36. Results

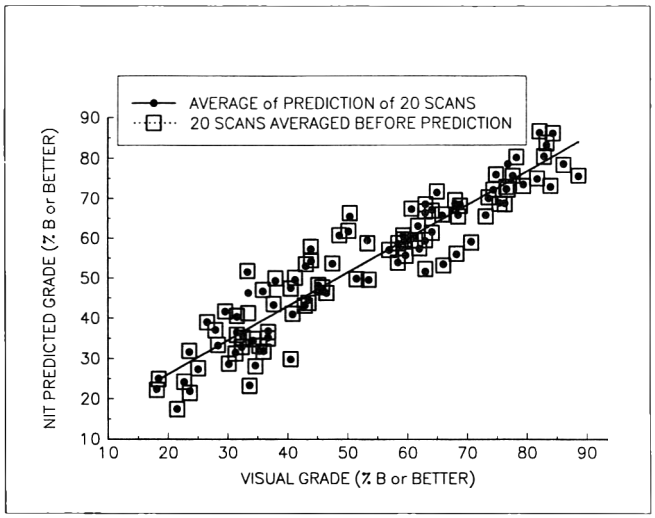


Fig. 6—Relationship between NIT predicted and Visual Grades of raisins for scans that were predicted and then averaged (r = 0.94, RMSE = 6.24) compared with scans that were averaged and then predicted (r = 0.93, RMSE = 6.23).

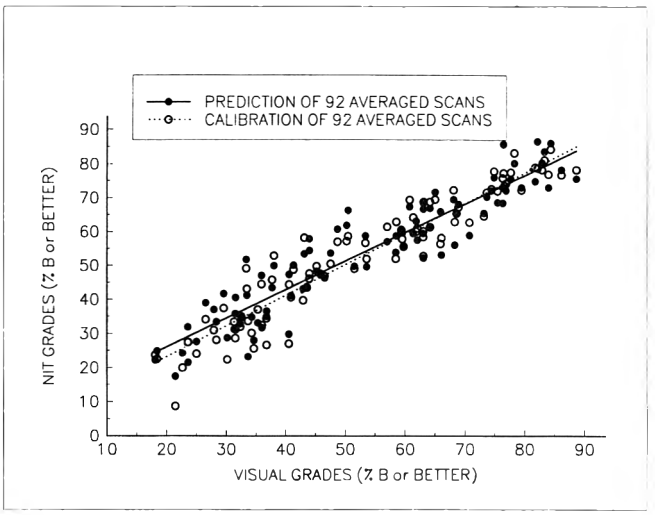


Fig. 7—Relationship between NIT grades and Visual Grades of raisins for scans that were averaged and predicted from a model, Model 1, (r = 0.93, RMSE = 6.23) and for an NIT calibration model, Model 2, of the same scans (r = 0.96, RMSE = 6.36).

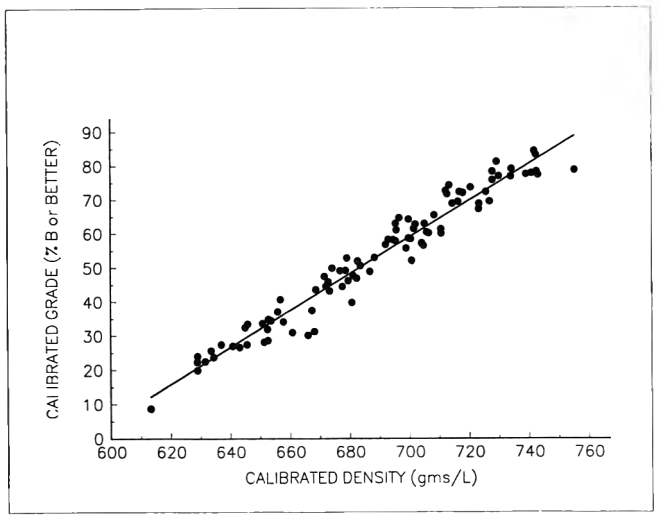


Fig. 8—NIT calibrated grades of raisins as related to NIT calibrated bulk density of raisins for 92 averaged scans. r = 0.98.

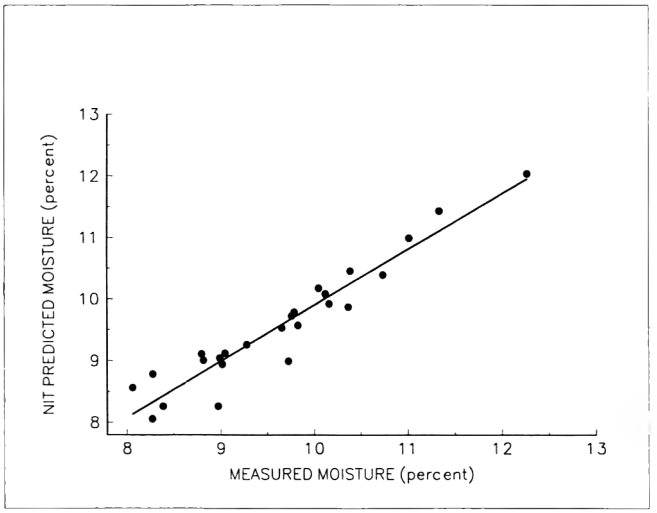


Fig. 9—Relationship of NIT predicted moisture and vacuum oven measured moisture of raisins. r = 0.96, RMSE = 0.30%.

of the prediction of the averaged scans were compared with those of a calibration model made with the same scans (Fig. 7). The values for the prediction model and the calibration were very close for most lots.

The NIT calibrated grade was related to the NIT calibrated bulk density (Fig. 8). This plot had a r = 0.98 and the RMSE = 4.01. This high correlation was expected because bulk density correlated with VG, and the measured grade and density had almost identical correlation with the first derivative of the spectra. Previous and current grading methods are linked to differences in densities of individual berries and bulk weights of the raisins. Thus we hypothesized that the PLS would yield calibrations for grade and density that were highly correlated.

Moisture

The moisture prediction model was used for 25 samples from the 1991 crop. Predicted results were compared with vacuum oven measured values (Fig. 9). For this prediction r = 0.96 and the RMSE = 0.30%. When the prediction model was used to predict the entire set of 1840 scans of the 92 lots, the variance between samples was 0.883, and the variance within samples was 0.041.

Results indicate that NIT could predict raisin moisture very well. This was expected because NIT has been reported as a very good predictor of moisture for a wide range of food and feed materials (Williams and Norris, 1987).

Near infrared analysis has been used to predict basic constituents, such as starch, protein, fiber, and sugars (Williams and Norris, 1987). The visual grade value of raisins would be related to some combination of such basic constituents. Once relationships between such constituents and grade was more completely determined NIT may predict basic constituents and grade. However, until such relationships are developed, any new grading system must make predictions in accordance with current grading standards.

A main difficulty in calibrating for subjective grade values is that reference values may change depending upon graders or panels. Visual grades would be expected to vary widely between graders or panels for lots that contain high levels of marginal quality raisins. The visual grading method measures discrete factors, but near infrared analysis results in average grade values. Also the B or Better category is very broad, and visual graders may assign the same numerical score to lots with similar weight percentages of C grade raisins regardless of overall quality of the B or Better fraction. NIT would also estimate the average quality of the B or Better fraction. This could lead to bias in the predictions. Analysis of many samples over several production seasons and growing conditions may reduce such variations.

Specific constituents that relate to raisin quality and that can be precisely and repeatedly measured by objective methods

would be preferred. NIT has the advantage that once a sample spectrum is determined many constituents may be predicted from it, provided proper and applicable calibrations are available.

The high correlation between subjective grade and measured bulk density can be very advantageous. To improve precision and accuracy for NIT prediction of grade, a large set of calibration samples must be included to represent all production variations, including season-to-season, that may be encountered. Because visual grade values may be highly variable for some lots, measured bulk density may be applicable to monitor visual grade and resolve discrepancies. During evaluation, if a wider than expected difference occurred between panel grade and NIT predicted grade, measured bulk density could be considered. Resolution of such differences for a large number of samples should lead to more accurate and precise NIT predictions.

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Names are necessary to report factually on available data; however, the USDA neither guarantees nor warrants the standard of the product, and the use of the name by USDA implies no approval of the product to the exclusion of others that may also be suitable.

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Optimization of Vapor Induced Puffing in Apple Dehydration

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ABSTRACT

Vapor induced puffing during high temperature fluidized bed (HTFB) dehydration of apple cubes was optimized using response surface methodology (RSM). Osmoblanching in sugar syrup prevented enzymatic browning. Successful puffing was achieved by creating a surface barrier to vapor by dipping the cubes in starch solution and manipulating the temperature and time of exposure in the HTFB. Optimum conditions for minimum bulk density with acceptable levels of nonenzymatic browning included dipping osmoblanched apple cubes in 1% starch solution, followed by HTFB drying 11 min at 157°C. Product bulk density exhibited a minimum while nonenzymatic browning values did not display an optimum. Minimum value of bulk density predicted by RSM and browning values were very close to those verified experimentally for product processed under the identified optimum conditions.

Key Words: apple dehydration, puffing, optimization

INTRODUCTION

SEVERAL PROCESSES have been used to dry apples but impaired product quality has restricted end use (Hall, 1989). Tissue shrinkage and cell collapse associated with water loss impaired product rehydration. A porous structure in air dried products has been obtained by using high temperature short time pneumatic dryers followed by conventional low temperature drying to a shelf stable moisture content (Jaramayan et al., 1980, 1982). To facilitate fluidization, Farkas et al. (1969), Lazar and Farkas (1971), and Brown et al. (1972) employed a centrifugal fluidized bed. Conventionally dried products may also be explosion puffed to impart a porous structure (Heiland et al., 1977; Sullivan et al., 1982). Explosion puffing has been successfully employed on apples (Eisenhardt et al., 1964; Sullivan et al., 1980), but the process requires specialized high-cost equipment.

High temperature fluidized bed (HTFB) drying has been successfully employed to simultaneously dry and puff osmotically pretreated rabbiteye blueberries (Kim and Toledo, 1987). This process considerably reduced dehydration time compared to conventional air dehydration.

Puffing is essential if a crispy texture is desired in a dried product. However, a multiplicity of sizes of internal pores may be achieved only if the product matrix resists shrinking as water is removed. High moisture materials like apples lack the type of solids matrix necessary to resist shrinking during conventional dehydration, therefore a special process must be developed to provide a crispy dried product. Tissue collapse during dehydration has been minimized by infusing solids or by applying an appropriate coating on the raw material surface prior to dehydration. Bobic et al. (1988) dipped raw products in a suspension of gelatinized starch to create a vapor impermeable layer on the surface. This coating retained vapor which developed adequate internal pressure to cause the solid to expand during fluidized bed drying. Jen et al. (1989) and Mudahar et al. (1989, 1990) used biopolymer infusion into carrots and potatoes to generate puffed HTFB dehydrated products with excellent rehydration properties. Torreggiani and Toledo (1991) osmoblanched apple cubes in syrup to prevent browning, and utilized HTFB dehydration to simultaneously dry and puff apple cubes. Blanching the apples in syrup removed moisture by osmosis and has been shown (Lerici et al., 1986) to control enzymatic browning without use of sulfites. Our previous work demonstrated the feasibility of HTFB drying for producing crispy dried apples, the process has not been optimized.

Response surface methodology (RSM) has gained acceptance in identifying optimum conditions in processes where several parameters and competing responses prevail. With HTFB dried apples, high temperature exposure favors puffing but also accelerates nonenzymatic browning. The fundamental aspects of RSM were extensively discussed in several reports (Box and Wilson, 1951; Davies, 1954; Hill and Hunter, 1966; Mead and Pike, 1975; Box et al., 1978; Thompson, 1982; Box and Draper, 1987). Our objective was to apply RSM to optimize vapor induced puffing of apple dices in a HTFB process.

MATERIALS & METHODS

Raw material

Apples (cv. Golden Delicious) were purchased locally. The fruits were peeled manually and diced into 10 mm cubes using a mechanical dicer (Ditto Dean Model TR-22, Los Angeles, CA).

Osmoblanching

One liter, 70° Bx low DE corn syrup (Sweetose 4300, A. E. Staley Co., Decatur, IL) was heated to 80°C on an electric hot plate. Apple dices (200g) in a wire mesh carrier were immersed into the hot solution, temperature was allowed to increase to 80°C. Five minutes after reaching 80°C, the carrier was rapidly removed, positioned under running cold tap water and the dices were rapidly rinsed 30 sec (Lerici et al., 1986). Dry matter content was increased and enzymatic browning was controlled by this procedure.

Dehydration

Conventional drying in a tunnel drier was employed to condition dices prior to high temperature fluidized bed (HTFB) treatment. The HFTB treated apples were also conventionally air dried to reach a final shelf stable moisture content. We employed a tunnel drier operated at 80°C and 4 m/s air velocity across the drying tray.

Simultaneous puffing and dehydration was achieved in an electrically heated HTFB operating at an air velocity of 15 m/s (Kim and Toledo, 1987). The HTFB temperature controller was pre-set at a higher level than the designated temperature to compensate for temperature drop on opening the door, introducing samples, and reclosing. Thus samples were immediately exposed to the desired temperatures. After sample introduction, the temperature controller set point was rapidly reset to the designated processing temperature. Samples were contained in a wire mesh carrier to facilitate rapid introduction and removal. Air temperature was continuously monitored and recorded by thermocouple. Because of the high air velocity, temperature rapidly stabilized and was maintained at the designated temperature by automatic control. No temperature peaks or valleys were evident in the temperature recordings during treatment.

Bulk density

This property, used as an index of puffing, was determined as weight/volume of puffed dices following Kim and Toledo (1987). All analyses were conducted in triplicate.

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Table 1—Coding of independent variables used in developing experimental data for optimization of a process for dehydration of apple dices in a high temperature fluidized bed (HTFB) drier

Independent variables	Symbols		Levels	
	Uncoded	Coded	Uncoded	Coded
Drying temp in HTFB, (°C)	T	X1	165	1
			155	0
			145	−1
Exposure time in HTFB, (min)	t	X2	18	1
			12	0
			6	−1
Concentration of starch, (%)	C	X3	3	1
			2	0
			1	−1
Pre-drying time, (min)	P	X4	30	1
			15	0
			0	−1

Table 2—Bulk density (BD) and browning (B) of HTFB dried diced apples for different coded values of treatment conditions (see Table 1)

Treatment no.[a]	Drying temp T	Exposure time t	Conc C	Pre-dry time p	BD	B
1	1	1	0	0	0.179	3.799
2	1	−1	0	0	0.265	0.223
3	−1	1	0	0	0.179	0.701
4	−1	−1	0	0	0.310	0.057
5	0	0	1	1	0.179	1.176
6	0	0	1	−1	0.194	0.320
7	0	0	−1	1	0.201	1.227
8	0	0	−1	−1	0.158	0.819
9	0	0	0	0	0.180	0.730
10	1	0	0	1	0.210	3.188
11	1	0	0	−1	0.161	0.716
12	−1	0	0	1	0.221	0.715
13	−1	0	0	−1	0.234	0.131
14	0	1	1	0	0.178	3.046
15	0	1	−1	0	0.185	3.097
16	0	−1	1	0	0.272	0.098
17	0	−1	−1	0	0.219	0.208
18	0	0	0	0	0.187	0.459
19	1	0	1	0	0.183	1.147
20	1	0	−1	0	0.179	1.024
21	−1	0	1	0	0.230	0.198
22	−1	0	−1	0	0.238	0.232
23	0	1	0	1	0.186	3.655
24	0	1	0	−1	0.174	1.660
25	0	−1	0	1	0.238	0.483
26	0	−1	0	−1	0.289	0.063
27	0	0	0	0	0.172	0.690

[a] Experimental runs were performed in random order.

Table 3—Regression coefficients of second order polynomials representing relationships between indicated response variables (Y_k) and independent variables of fluidized bed temperature (i or j = 1), exposure time (i or j = 2), concentration of starch (i or j = 3) and pre-drying time (i or j = 4)

Coefficients	Bulk density BD k = 1	Browning B k = 2
bk0	0.179	0.626
bk1	−0.019	0.672
bk2	−0.043	1.235
bk3	0.005	−0.052
bk4	0.002	0.561
bk11	0.023	0.037
bk21	0.011	0.733
bk22	0.034	0.657
bk31	0.003	0.039
bk32	−0.015	0.015
bk33	0.001	0.095
bk41	0.015	0.472
bk42	0.016	0.394
bk43	−0.014	0.112
bk44	0.005	0.290

Extent of nonenzymatic browning

Nonenzymatic browning was measured based on color intensity of aqueous methanol extractable pigments (Mowlah, et al., 1983). Browning was expressed as optical density at 420 nm/g dry matter. All analyses were conducted in triplicate.

Table 4—Analysis of variance showing significance of effects of processing variables on bulk density and browning

Process variables	DF	Sum of squares: Bulk density BD	Sum of squares: Browning B
Drying temp (T)	2	0.008***	8.658***
Exposure time (t)	2	0.030***	23.100***
Conc. of starch (c)	2	0.002	0.089*
Pre-drying time (p)	2	0.003*	5.789***

Significant at * 10%; *** 1% level.

Table 5—Analysis of variance showing effects of treatment variables as linear or quadratic terms and interaction (cross product) effects on response variables, bulk density and browning

Source	DF	Sum of squares: Bulk density BD	Sum of squares: Browning B
Model	14	0.039**	33.922**
Linear	4	0.027**	27.547**
Quadratic	4	0.008**	2.657*
Cross Product	6	0.004*	3.718*
Residual	12	0.003	1.844
Lack of fit	10	0.003	1.801**
Pure error	2	0.000	0.043
% Variability explained (R^2)		93.03	94.84

Significant at * 5% level; ** 1% level

Preliminary drying studies

Prior to conducting the RSM experimental design, the relevance and range of appropriate variables were identified in preliminary experiments. We established that diced apples did not puff under conditions reported by Mudahar et al. (1989, 1990). We partially dehydrated osmoblanched apple dices in a conventional dryer and dipped them in starch solution. These dices were then exposed to HTFB, and dried to a final shelf stable moisture in a conventional drier. We evaluated the product for bulk density and crunchiness (Torreggiani and Toledo, 1991). After osmoblanching, the following treatments were used to precondition the diced apples for HTFB drying: osmo-dehydration in 70°C Bx corn syrup at 25°C for 24 hr; conventional drying for 20, 30 and 40 min at 80°C; osmo-dehydration for 24 hr at 25°C and 1 atm. in 70° Bx corn syrup containing 3% of a biopolymer mixture (maltodextrin:pectin, 1:9); osmo-dehydration for 24 hr at 25°C and 25 mm Hg absolute pressure in 40° Bx corn syrup containing the biopolymer mixture; and dipping for 2 min. in 2% gelatinized tapioca starch.

RSM experimental design

After the preliminary experiments established conditions under which puffing of apple dices occurred, RSM was used to optimize the process. A 3-level 4 factor design was adopted (Box and Behnken, 1960). The response variables (Y_k), bulk density (BD) and browning (B) were represented as a function (f_k) of four independent processing variables, namely: HTFB temperature (T); HTFB exposure time (t); concentration of starch in dip solution (c) and pre-drying time in the conventional drier (p).

$$y_k = f_k (T, t, c, p) \quad (1)$$

Second degree polynomials were used for f_k.

$$y_k = bk_0 + bk_iX_i + bk_{i,j}X_iX_k \quad (2)$$

Subscripts i or j = 1 represent HTFB temperature, 2 = HTFB time (see Table 1), 3 = starch concentration in the dip solution, and 4 = pre-drying time. Independent variables were coded based on a linear functionality between coded and actual values.

Statistical analysis

The Statistical Analysis System (SAS Institute, Inc., 1985) was used to fit the second order polynomial equation to experimental data to obtain the coefficients of Eq. (2).

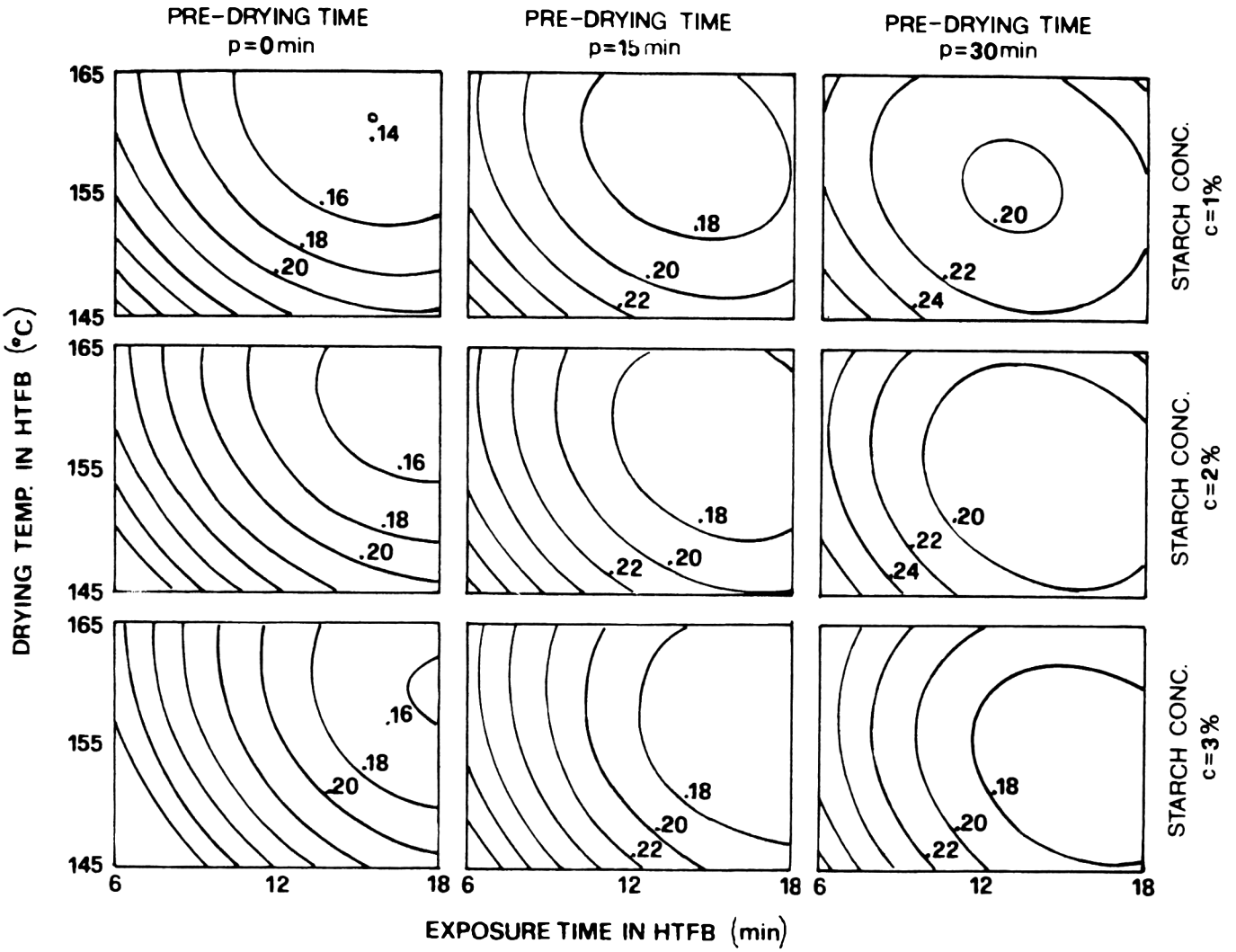


Fig. 1—Contour plots of bulk density (BD) at different levels of independent variables during dehydration in a high temperature fluidized bed (HTFB).

Verification

Optimum conditions were identified by RSM. Dried apple dices were prepared under those conditions and values of bulk density and browning were compared to those predicted by RSM model equations.

RESULTS AND DISCUSSION

Preliminary drying experiments

The preconditioning treatments on osmoblanched apple dices prior to HTFB exposure were designed to prevent the puffed structure from collapsing after removal from the fluidized bed. The primary osmoblanching step increased dry matter from the 9 to 12% in raw apples to 23 to 25%. The second osmodehydration step increased dry matter further to 34 to 35%. Twenty and 40 min conventional air drying following primary osmoblanching increased dry matter to 35% and 45%, respectively. Secondary osmodehydration in 70° Bx corn syrup at 1 atm or 40° Bx corn syrup under vacuum with biopolymers raised dry matter to 40 to 42%. Bulk densities of the dried apples were 0.238 to 0.310 g/cm^3 when osmoblanched only and exposed to HTFB; 0.201 to 0.195 g/cm^3 when preconditioned after osmoblanching by osmodehydration or conventional drying prior to HTFB exposure; 0.169 to 0.171 g/cm^3 when preconditioned by osmodehydration in corn syrup and biopolymers prior to HTFB exposure; and 0.155 to 0.158 g/cm^3 when dipped in starch solution prior to HTFB exposure. Bulk density was increased slightly by preconditioning osmoblanched apples to elevate dry matter prior to HTFB treatment. However, the lowest bulk density was obtained when the osmoblanched apples were dipped in starch solution prior to HTFB exposure. A puffed appearance was not evident when HTFB temperature was below 145°C regardless of HTFB exposure time. Product attributes were acceptable when osmoblanched apples were dipped in starch solution, exposed 12 min to HTFB at 150°C and dried to 5% moisture in a conventional dryer at 80°C. The most readily discernible quality attributes were lack of shrinkage and color, quantitatively represented by bulk density and browning, respectively. Puffing manifested by reduced bulk density, was necessary for the dried product to be crunchy. The range of values of independent variables in the RSM experimental design was based on these results.

RSM experiments

Browning and bulk density data from the RSM experimental design are shown in Table 2. Regression coefficients of the second degree polynomial expression for browning and bulk density were calculated (Table 3).

Bulk density was affected most by HTFB temperature, HTFB exposure time and their interactions with starch concentration in the dip and pre-drying time. These terms had the highest coefficients (Table 3). Browning was affected by all independent variables except starch concentration in the dip. In addition, starch concentration interactions also had very little effect on browning. The ANOVA table (Table 4) showed a lack of significance of effect of starch concentration in the dip on either

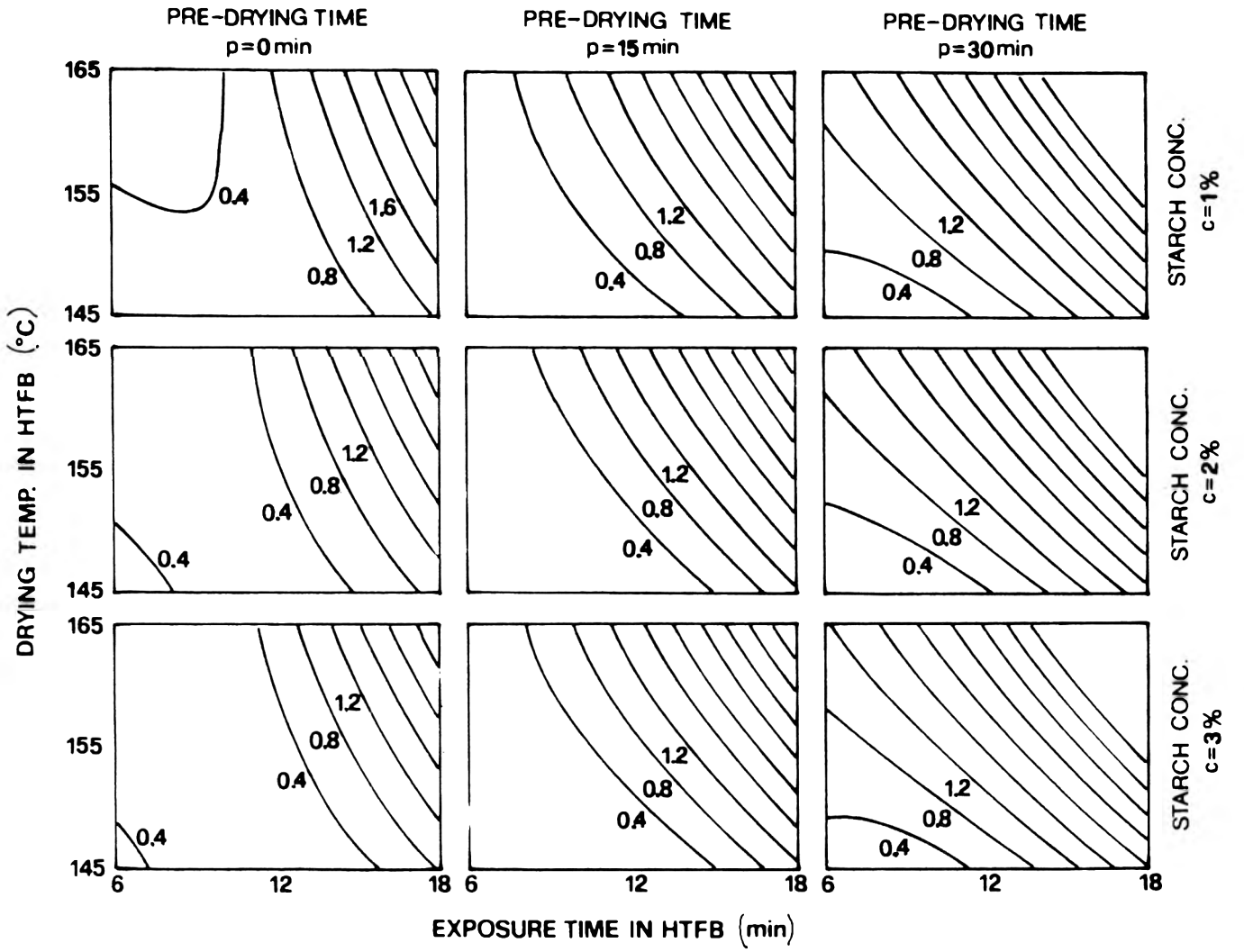


Fig. 2—Contour plots of browning (B) at different levels of independent variables during dehydration in a high temperature fluidized bed (HTFB).

bulk density or browning. Results indicate that the lowest starch concentration in the experimental design was adequate to induce puffing therefore increasing concentrations resulted in minimal bulk density changes. Starch was not important in color development while HTFB temperature, time, and initial moisture of preconditioned dices prior to HTFB exposure all were important in browning. Hendel et al. (1955), demonstrated the dependence of moisture content, and temperature on rate of browning development in dehydrated vegetables.

Preliminary experiments showed that dipping in starch solution was necessary in producing a puffed product. A vapor impermeable dried starch film on the surface of the dices allowed water vapor pressure to build-up inside the dices during HTFB exposure which made the dices expand. This hypothesis was supported by the strong interaction between starch concentration and exposure time in the expression for bulk density.

An ANOVA table for effects of treatment variables as linear, quadratic, or interaction coefficients on response variables browning and bulk density was developed (Table 5). The model represented by Eq. (2) for BD showed no significant lack of fit and coefficient of determination for the regression was 0.93. Data on browning on the other hand, showed a significant lack of fit. The coefficient of determination of the regression was 0.95. Box and Draper (1987) attributed significance in lack of fit to indicate a model which may not have included all appropriate functions of independent variables. Although the significant lack of fit may be corrected by appropriate transformation of independent variables, we were unsuccessful in generating nonsignific-

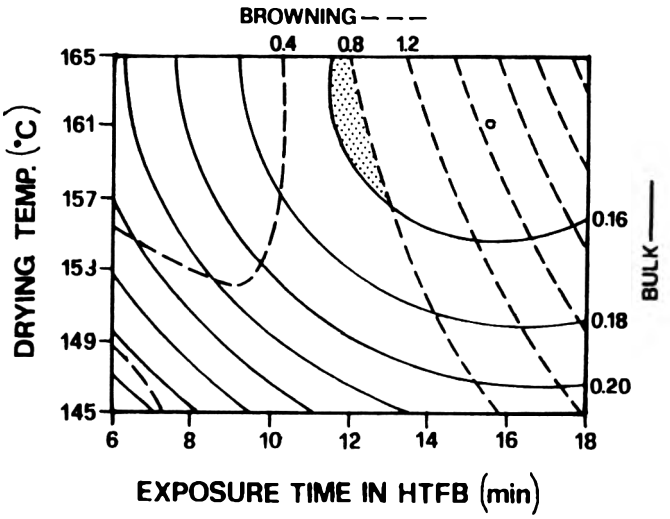


Fig. 3—Superimposed plots of bulk density (BD) and browning (B) at 1% starch concentration and 0 min pre-drying time.

ance. The experimental region may be too large for the quadratic model we used. Box and Draper (1987) pointed out however, that when a large amount of data were included in the analysis, a model with a significant lack of fit could still be used. However, the results should be later verified experimentally and good

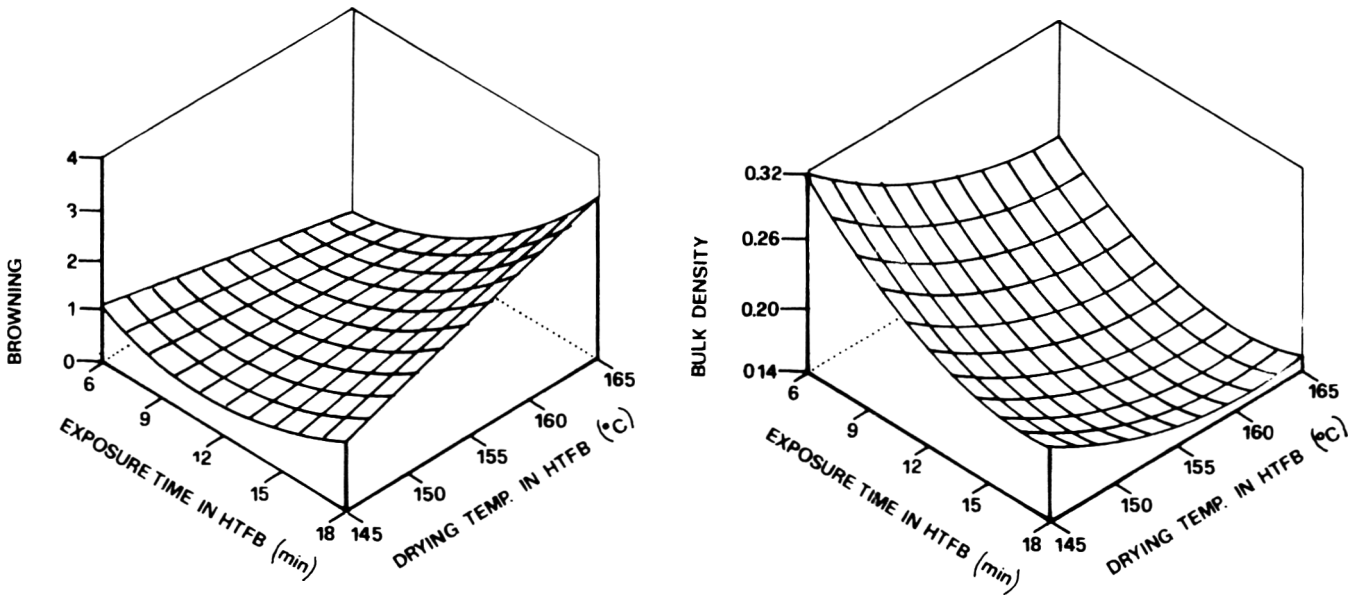


Fig. 4—Response surfaces of bulk density (BD) and browning (B) at constant starch concentration of 1% and 0 min pre-drying time.

agreement must exist between values calculated using the model equation and the experimental value for the response variable at the point of interest. We considered the high coefficient of determination between browning and independent variables as evidence of the applicability of the model within the range of variables included.

The procedure described by Myers (1971) and Draper (1963) was used to locate stationary points in the four-dimensional space formed by the independent variables. Bulk density exhibited a minimum (BD = 0.148 g/cm^3 at T = 161.5°C and t = 15.06 min when starch concentration in the dip was minimum (1%), and no preconditioning step occurred between osmoblanching and HTFB. Browning exhibited no optimum. On the contrary, browning increased with both HTFB temperature and exposure time. At any combination of HTFB temperature and exposure time, browning was least when dip starch concentration was 3% and no pre-drying was used.

Nine contour plots were developed for bulk density (Fig. 1) and browning (Fig. 2). In this product, bulk density was more important as a quality attribute than color, since adequate puffing was necessary for a crunchy dried product. Adequately puffed dices also have better appearance because of the absence of shriveling or warping of the surface. Browning also affects appearance of the product and should be minimized. However, in some applications such as breakfast cereals, a mild caramelized flavor associated with light browning may complement the apple flavor.

Optimization of the process was based on minimum bulk density and a reasonable extent of browning. The superimposed contour plots for bulk density and browning were compared (Fig. 3). The shaded area represents a region of minimum bulk density and browning at 0.8 units. The optimum condition for this process was a dip in 1% starch solution of osmoblanched diced apples without preconditioning and HTFB drying at 157–165°C for 11 to 13 min. Response surfaces for BD and B (Fig. 4) showed a slight trough in the response for B relative to HTFB exposure time but B increased almost linearly with temperature at all exposure times. Response surface for BD on the other hand was concave with a minimum. However, the point of minimum BD value was outside the optimum range of conditions because the color value exceeded 0.8 units.

The optimum processing conditions identified by RSM (157°C, 13 min) were used to process a batch of dried apples. BD of the product was 0.158–0.162 (mean, 0.16) g/cm^3 compared to 0.16 g/cm^3 identified as optimum by RSM. Color value was 0.78–0.82 (mean, 0.8) compared to 0.8 determined by RSM.

CONCLUSIONS

PUFFING OF DRIED APPLES in a HTFB was optimized by RSM. The optimum process involved osmoblanching raw apples to increase the dry matter to 23–25% followed by dipping in 1% gelatinized starch solution, exposure to HTFB at 157°C for 11 min. and final drying to 5% moisture in a conventional drier at 80°C.

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Limonoid Glucosides in Fruit, Juice and Processing by-products of Satsuma Mandarin (*Citrus unshiu* Marcov.)

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ABSTRACT

In a study on improving utilization of waste from processing of Satsuma mandarin (*Citrus unshiu* Marcov.) the limonoid glucosides of fruit, juice, and by-products were measured using HPLC and TLC. All materials had the 17-β-D-glucopyranoside derivatives of limonoids reported in other commercial citrus fruit. Citrus molasses was a good source for industrial scale extraction of limonoid glucosides. An extraction system using polystyrene divinylbenzene resins, was developed which could be expanded to industrial scale.

Key Words: mandarin, limonoid glucosides, citrus molasses

INTRODUCTION

THE UTILIZATION OF CITRUS PROCESSING WASTE is increasingly important. New technologies, such as supercritical fluid extraction and preparative chromatography applied to food processing have improved extraction and isolation of chemical constituents of food (Braddock and Cadwallader, 1992).

Limonoids are one group of the two bitter principles in *Citrus*. Among them, limonin is the major cause of limonoid bitterness in a variety of citrus juices. Limonoid research has been focused toward determining ways to remove bitter limonoids from citrus products (Hasegawa and Herman, 1992; Hasegawa et al., 1992). The commercial demand for limonoids has increased considerably since they were found to exhibit chemopreventive activity against cancer in laboratory animals (Lam et al., 1994; Miller et al., 1994) and antifeedant activity against certain insects and termites (Klocke and Kubo, 1982; Serit et al., 1991).

Limonoids also occur as glucoside derivatives in mature citrus fruit tissues and seeds. Twenty-one limonoid glucosides have been isolated and characterized. All of those were monoglucosides in which one limonoid molecule is linked with one D-glucose molecule at the 17-position of the open limonoid D-ring by a β-glucosidic linkage as in, for example, limonin 17-β-D-glucopyranoside (LG). Limonin glucoside was shown to exhibit similar chemopreventive activity against cancer in laboratory animals (Miller et al., 1994). Limonoid glucosides are almost tasteless and are water soluble, whereas some limonoid aglycones are extremely bitter and have very low solubility in water. Thus, limonoid glucosides would be ideal functional food additives, and it may be advantageous to recover such compounds from citrus processing wastes for other uses (Hasegawa et al., 1994).

Satsuma mandarin (*Citrus unshiu* Marcov.) cultivation originated in Japan. Today they are grown in South Korea, China, Mediterranean costal countries and Spain as well as the U.S. Satsuma mandarins are the most popular fresh citrus fruits and processed juice products consumed in Japan. The annual production was 1.5 million metric tons in 1993. Concentrations of limonoid aglycones in the juices from mature Satsuma mandarin are generally low, thus the limonoid bitterness problem occurs infrequently (Hashinaga et al., 1977; Izumi et al., 1981).

Our objectives were to follow the changes in LG, the predominant limonoid glucoside in citrus fruit tissues, during Satsuma mandarin fruit development. We then analyzed the limonoid glucosides of processed juice products and processing by-products of Satsuma mandarin, to evaluate suitability as sources for limonoid glucosides recovery. Commercially debittered and deacidified juices of Hassaku (*Citrus hassaku*) were also analyzed. An extraction experiment was conducted with resins commonly used for commercial debittering of citrus juices as a basis for developing an industrial scale system to recover limonoid glucosides for other uses.

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MATERIALS & METHODS

Materials

Frozen concentrated juice samples were obtained from the Momoyama processing plant of Wakayama Prefectural Agricultural Cooperatives from October 1990 through February 1991. Juices were processed with an FMC In-Line juice extractor. Processed juices and by-product samples, including single strength juice, concentrated juice, finisher pulp, centrifugal pulp, intact peel residue, pressed peel residue, dried peel residue and citrus molasses, were obtained from the Momoyama plant during the 1992 processing season. Commercially debittered and native Hassaku juices were also obtained from the Momoyama plant.

Fruit samples were obtained 11 times between July 20 and December 23, 1992, from two trees grown at a commercial orchard in Shimotsu, Wakayama, Japan. For each sample date, 10 fruits were picked from each tree. All reported values are expressed as averages from the lots of fruits.

A C_{18} reverse phase analytical HPLC column, TSK-gel ODS120T, was purchased from Tosoh Corp. (Tokyo, Japan). Silica gel TLC plates, SIL G-25, was purchased from Macherey-Nagel (Germany). SEP-PAK® plus C_{18} Environmental Cartridge was purchased from Millipore Corp. (Milford, MA). Industrial resins, HP-20 and WA-30 were obtained from Nihon Rensui Co. Ltd. (Tokyo, Japan). All other chemicals were purchased from Nacalai Tesque (Kyoto, Japan).

Preparation and extraction of fruit samples

Flesh and peel of sample fruit were separated by hand. The contents of limonoid glucosides of the peel and flesh were analyzed by the method of Fong et al. (1992). A portion of each sample was blended in methanol with a Waring blender (New Hartford, CT). For analysis, 5 g of slurry was further homogenized with a Polytron tissue homogenizer (Kinematica, Switzerland) with 20 mL methanol. The homogenate was centrifuged at 9,000 *g* for 10 min. The residue was extracted twice with 20 mL of 70% methanol. The extraction procedure was repeated three times. The combined extract was evaporated to dryness and dissolved in minimal volume of H_2O. The extract was then passed through a C_{18} SEP-PAK. The SEP-PAK was activated with 3 mL of methanol and rinsed with 10 mL of H_2O, before sample loading. The cartridge was then washed with 10 mL of H_2O and limonoid glucosides were eluted with 6 mL of methanol. The eluate was evaporated and the residue was dissolved in 1 mL of methanol.

Preparation and extraction of liquid samples

Limonoid glucosides of all liquid samples, including single strength juice, concentrated juice, finisher pulp and citrus molasses, were extracted by the modified method of Fong et al. (1990) as follows. Con-

Table 1—Limonoid glucosides of Satsuma mandarin frozen concentrated juices produced in 1990 season[a]

Date	Limonoid glucosides concentrations (ppm)[b]				
	LG	NG	NAG	OG	Total
Oct. 20, 1990	124	47	41	0.7	213
Oct. 30,	133	56	47	0.7	237
Nov. 10,	140	63	43	0.5	247
Nov. 20,	139	59	44	0.6	242
Nov. 30,	146	69	50	0.8	266
Dec. 10,	141	63	48	0.6	253
Dec. 20,	134	44	39	0.3	217
Jan. 11, 1991	113	30	34	0.1	177
Jan. 19,	112	39	38	0.2	189
Feb. 1,	123	48	45	0.2	216
Feb. 15,	114	36	32	0.1	182
Mean	129	50	42	0.4	223
S.D.	12	12	5	0.2	28

[a] Values show the converted rate of 9.0° Brix.
[b] L, Limonin; N, Nomilin; NA, Nomilinic acid; O, Obacunone; G, 17-β-D-glucopyranoside.

centrated juices were reconstituted to 9.0° Brix with distilled water prior to extraction. Each liquid sample (10g) was mixed with 20 mL of 70% methanol. For single strength juices and reconstituted juices 10 mL of each sample was used. The mixture was then homogenized with the Polytron, and the homogenate was centrifuged as described. The precipitate was extracted twice with 30 mL of 70% methanol. The combined supernatants were evaporated and the limonoid glucosides were isolated with SEP-PAK as described. For molasses samples, two SEP-PAKs were stacked. The stacked SEP-PAKs were preconditioned with 6 mL of methanol and rinsed with 20 mL of H_2O. Limonoid glucosides were eluted with 12 mL of methanol.

Preparation and extraction of solid by-products

Limonoid glucosides of solid samples including centrifugal pulp, intact peel residue, pressed peel residue and dried peel residue, were extracted with 70% methanol. Each solid (10g) was used. The extraction was carried out by methods described for liquid samples. The extracts were evaporated to remove methanol. The aqueous portion was passed through a SEP-PAK, and eluted with methanol. For dried peel residue, stacked two SEP-PAKs were used. Eluates were evaporated to dryness and dissolved in 1 mL of methanol.

Analyses

Limonoid glucoside contents of sample extracts were analyzed by HPLC. A LC6A HPLC system (Shimadzu Co.) was used. The sample (5μL) was injected onto a C_{18} reverse-phase analytical column, using an automatic injector AS-8010 (Tosoh Corp.). When necessary, the sample was diluted up to tenfold with H_2O, prior to injection. The column was eluted with a linear gradient system starting with 15% acetonitrile and ending with 26% in 3 mM H_3PO_4 in 40 min. Flow rate was 1 mL/min and the eluate was monitored by UV absorption at 210 nm (Ozaki et al., 1991). Peaks were identified by the method of Herman et al. (1990), and quantified by comparison of peak areas with standards. Standards of limonoid glucosides were isolated from citrus seed and characterized by NMR spectroscopy (Hasegawa et al., 1989; Bennett et al., 1989). All analyses were performed in duplicate. TLC analyses were also done to confirm results and estimate concentrations when the resolution of HPLC chromatograms was poor (Fong et al., 1990; Ozaki et al., 1991).

Purification of limonoid glucosides

Purification of limonoid glucosides from molasses of Satsuma mandarin was performed using industrial resins. An adsorbent, HP-20, and a weak base anion exchange resin, WA-30, were tested. These are polystyrene divinylbenzene resins and are commonly used for commercial debittering and deacidification of Hassaku juices in Japan. Each resin (500mL) was packed in a glass column (ϕ 26.4 × 1000 mm). The HP-20 column was conditioned by washing with 3L of methanol prior to washing with 3L of distilled water.

Satsuma mandarin molasses (45° Brix) was diluted to 15° Brix with water and clarified by centrifugation at 1,000 × *g* for 10 min. Diluted and clarified molasses (1L) was adjusted to pH 4.5 and applied to the HP-20 column. The column was washed thoroughly with water and eluted with 1L of methanol. The eluate was evaporated and dissolved in minimal volume of water, then pH was adjusted to 6.5.

The WA-30 column was conditioned with 3L of 0.5N NaOH followed by 3 L of 0.5N HCl. The HP-20 eluate was applied to the WA-30 column. The column was washed thoroughly with water and eluted with 1L of 0.5M NaCl. The eluate was re-applied to the HP-20 column and subsequently eluted with methanol to remove salts. The final eluate was evaporated and dissolved in 30 mL of methanol. The flow rate for all columns was 10 mL/min.

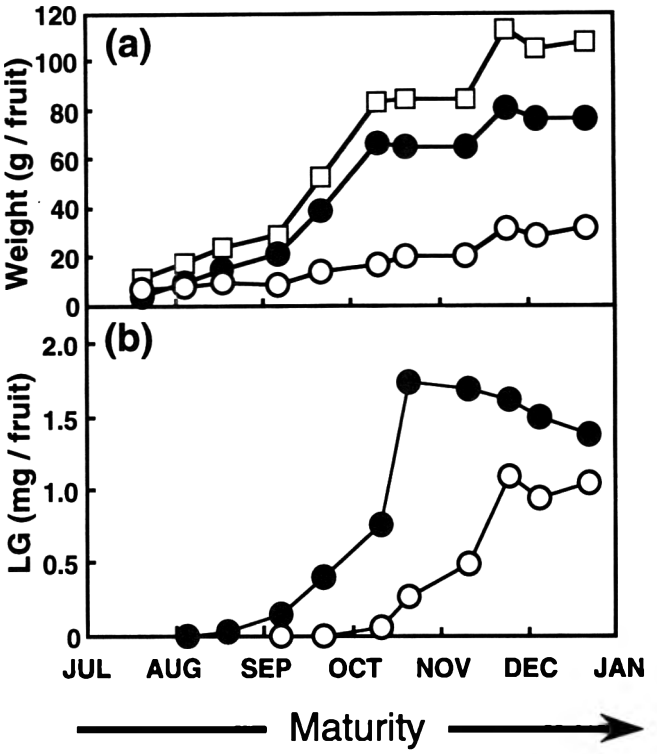


Fig. 1—Changes in LG contents of Satsuma mandarin during fruit development. □—□, Whole fruit; ●—●, Flesh ○—○, Peel.

RESULTS & DISCUSSION

Frozen concentrated juices

We first analyzed Satsuma mandarin frozen concentrated juices for limonoid glucosides and found that they contained very high concentrations, including the 17-β-D-glucopyranosides of limonin, nomilin (NG), nomilinic acid (NAG) and obacunone (OG). Trace amounts of glucosides of deacetylnomilin (DG) and deacetylnomilinic acid (DAG) were also present. These limonoid glucosides have been commonly found in most commercially grown citrus fruit.

Results from 11 sample analyses showed that the Satsuma mandarin frozen concentrated juices contained an average of 223 ppm of total limonoid glucosides (Table 1) and ranged from 182–266 ppm. This average concentration was lower than that of commercial orange juices sold in the US, but higher than that of grapefruit and lemon juices (Fong et al., 1990). As expected, LG was the predominant limonoid glucoside in all juice samples, averaging 129 ppm or 58% of the total. This was in agreement with values reported in other citrus juices. LG contents in orange, grapefruit, and lemon juices have been reported as 56, 63 and 66% of the total, respectively. The analytical values of limonoid glucosides in juices were fairly consistent throughout the 1990 processing season. This was in contrast to the report of Hasegawa et al. (1991) which stated that limonin glucoside contents of navel orange and Valencia orange (Fong et al., 1992) increased as fruit maturation progressed.

Fruit

Changes in fruit weight during the course of the experiment were followed (Fig. 1a). At least sharp increases in fruit weight

Table 2—Limonoid glucoside composition of mature Satsuma mandarin fruit[a]

Tissues	Concentration of limonoids (ppm)			
	LG	NG	NAG	OG
Flesh	186	144	126	6
Peel	360	131	0	11

[a] Averages of December samples (Dec. 7 and 23).

Table 3—Concentrations of limonoid glucosides in Satsuma mandarin juice processing products

Samples	Limonoid glucosides (ppm)				
	LG	NG	NAG	OG	Total
Whole Fruit	237	159	88	7.2	491
Juice Products:					
Single Strength Juice	99	41	72	2.3	215
Finisher Pulp	112	62	28	1.8	204
Centrifugal Pulp	173	57	61	2.6	294
Residue Products:					
Intact Peel Residue	359	235	168	7.0	769
Pressed Peel Residue	488	281	153	58	980
Dried Peel Residue	—	—	—	—	3561[a]
Press Liquor	255	120	100	60	535
Molasses	—	—	—	—	2486[a]

[a] Values show estimations by TLC analyses.

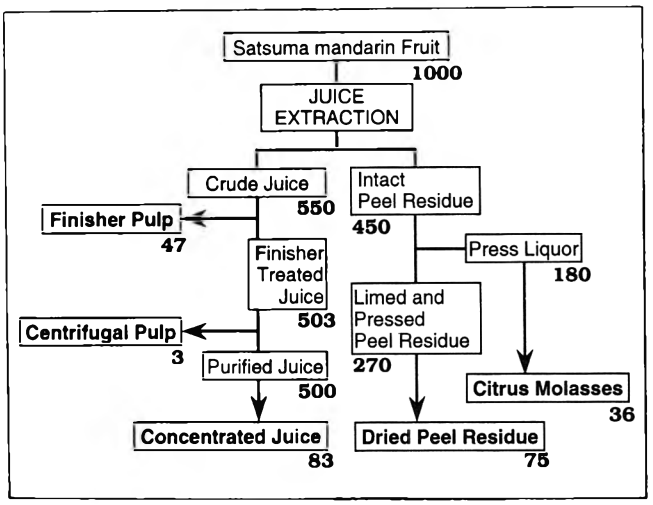


Fig. 2—Steps in Satsuma mandarin juice processing. Estimated amounts from 1000 kg fruit.

occurred during the course of fruit development. The first occurred between September and October, mainly due to an increase in flesh weightt. The second occurred between November and December where sharp increases in both flesh and peel weight were observed. Generally, the late ripening type of Satsuma mandarin is fully matured and ready for harvest in late November in the Shimotsu area.

Changes in LG contents of flesh and peel during fruit development were also followed (Fig. 1b). LG was first detected at the beginning of August in the flesh and at the beginning of September in the peel. The LG content of flesh increased sharply from the middle of September and reached a maximal level by the middle of October, and remained at that level until harvest season. Interestingly, LG content of peel sharply increased in November where the sharp increase in peel weight was observed. This synchronized increase between tissue weights and LG content suggests that the biosynthesis of LG is strongly related to tissue maturity. In a mature fruit, the content of LG was 17 mg in flesh and 11 mg in peel. These values were similar to those for Valencia oranges grown in the US (Fong et al., 1992).

The limonoid glucoside composition of mature Satsuma mandarin fruit was determined (Table 2). Glucoside proportions in Satsuma mandarin fruit were similar to those in frozen concentrated juices. However, concentrations of total limonoid glucosides, 460 ppm in flesh and 500 ppm in peel, were about twice those from the concentrated juice analyses. This difference between raw fruit and processed products indicated that large amounts of limonoid glucosides were retained in the processing waste. We thus analyzed processing wastes to evaluate suitability as sources of limonoid glucosides.

Table 4—Estimated yield of limonoid glucosides from 1000 kg of Satsuma mandarin fruit

By-products	Estimated Total Limonoid Glucosides (g)
Fruit	500
Finisher pulp	10
Centrifugal pulp	1
Concentrated juice	120
Citrus molasses	90
Dried peel residue	180

Table 5—Limonoid glucosides in commercially debittered and deacidified Hassaku juices

Samples[a]		Limonoid glucosides (ppm)				
		LG	NG	NAG	OG	Total
Treated	Mean	55	7	19	—	81
	S.D.	9	3	4	—	14
Untreated	Mean	146	80	109	5	340
	S.D.	22	17	22	2	30

[a] 20 treated samples and 10 untreated samples were analyzed.

Processed juice products and by-products

Concentrations of limonoid glucosides in the products of juice processing were compared (Table 3). Analyses were conducted weekly throughout the 1992 processing season, from Nov. 11, 1992 to Feb. 1, 1993. For dried peel residue and molasses, the values were estimated by TLC, because of low resolution by HPLC.

The total limonoid glucoside concentration of single strength juice was 215 ppm. This was consistent with results from the previous year. The concentrated juice contained $>$ 1400 ppm of glucosides while the pulp contained $<$ 300 ppm. The limonoid content of whole fruits sampled from the juice plant confirmed that of fruit collected from the orchard. The residue products contained high concentrations of limonoid glucosides. The dried peel residues contained $>$ 3500 ppm while the molasses contained $\approx$2500 ppm.

The composition of whole fruit and press liquor were quite similar except for concentrations of obacunone glucoside. This glucoside in the press liquor was eightfold higher than that in the fruit. This may be due to conversion of nomilin glucoside into obacunone glucoside during liming of the peel. Nomilin glucoside is the most unstable limonoid glucoside and such conversion has been reported (Miyake et al., 1991).

A typical juicing procedure of Satsuma mandarin fruit during processing showing material balances (Kohno, 1978) was summarized Fig. 2. When 1,000 kg of Satsuma mandarin was processed, 83 kg of concentrated juice, 47 kg of finisher pulp, and 3 kg of centrifugal pulp were obtained. The peel residue from juice extraction after liming, pressing, and drying produced 75 kg of dried peel residue and 36 kg of citrus molasses.

Based upon concentration data yields of limonoid glucosides from 1,000 kg of Satsuma mandarin fruit were estimated (Table 4). About 500 g of total limonoid glucosides would be present in 1000 kg of Satsuma mandarin fruits. Only about 120g is retained in the concentrated juice, (about 24%). Dried peel residue contains 180g, (36% of the total) and citrus molasses contains about 90g (18% of the total). These two by-products should provide good prospects for recovering limonoid glucosides. However, citrus molasses would be a better source for

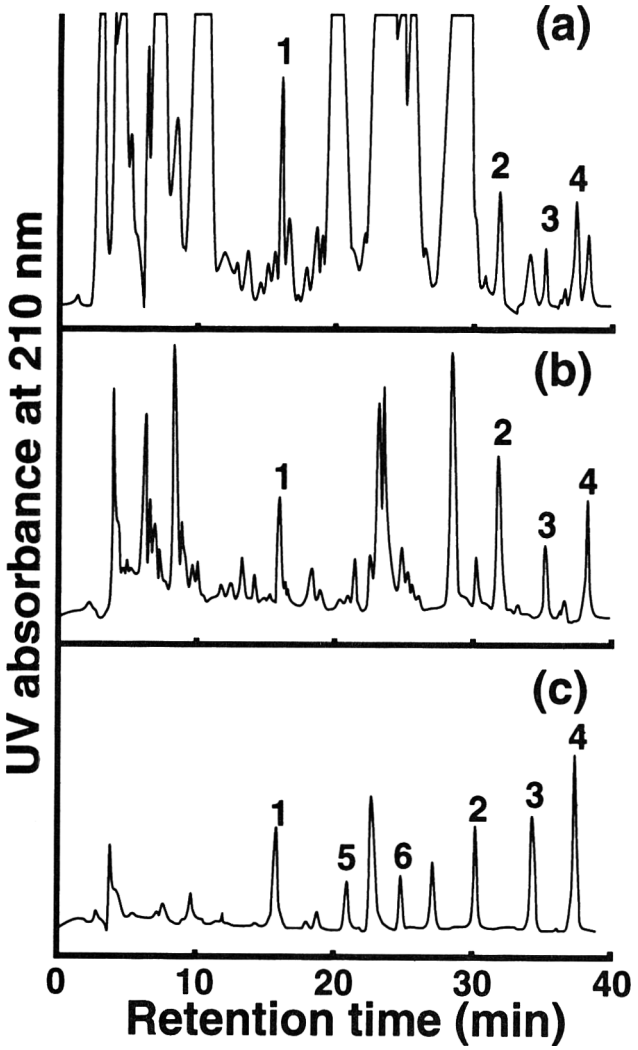


Fig. 3—Typical HPLC chromatograms from purification steps. (a) Crude molasses; (b) HP-20 treated; (c) WA-30 treated. (1) limonin-G; (2) nomilin-G; (3) nomilinic acid-G; (4) obacunone-G; (5) deacetylnomilinic acid-G; (6) deacetylnomilin-G; G, 17-β-D-glucopyranoside.

Table 6—Purification of limonoid glucosides from Satsuma mandarin molasses (Lab-scale system)

Process	Limonoid glucosides (mg)					Weight[a] (g)	Purification (-fold)	Yield (%)
	LG	NG	NAG	OG	Total			
Crude molasses	199	104	87	54	444	220	1	100
HP-20 treated	142	75	61	42	320	2.5	63	72
WA-30 treated	66	39	37	89	231	0.4	286	52

[a] Weight of solid after evaporation, except crude molasses.

recovery, since it is in liquid form and an extraction step could be omitted.

Commercially debittered and deacidified Hassaku juices

Hassaku fruit is commercially processed into juices which are often debittered and deacidified (in Japan). Limonoid glucosides concentrations in such commercially debittered and deacidified Hassaku juices were determined (Table 5). Twenty debittered and deacidified samples and 10 untreated samples were analyzed. Whereas the averaged concentration of total limonoid glucosides in untreated samples was 340 ppm, that in treated was 80 ppm. This clearly showed that most of the limonoid glucosides were removed during the process. Since the HP-20 resin for debittering was capable of absorbing limonoid aglycones, possibly limonoid glucosides were also absorbed to the resin. The limonoid glucosides retained on the resin should be recovered for other uses and the treated resin could be an excellent source of limonoid glucosides.

Lab-scale purification

We designed a system for recovery and purification of limonoid glucosides from citrus molasses using HP-20 and WA-30 columns. Typical HPLC chromatograms were compared for each purification step (Fig. 3). Satsuma mandarin molasses was passed through the HP-20 column which retained the limonoid glucosides. In this step, highly hydrophilic compounds in the molasses, such as sugars, passed through the column, whereas hydrophobic compounds including polyphenols were retained.

WA-30 column was then used to separate the limonoid glucosides from the phenolic compounds. As expected, limonoid glucosides were retained on the anion exchange column in the pH region used. A portion of limonoid glucosides was most likely retained by adsorption (Manlan et al., 1990). The phenolic compounds were also retained on the resin and eluted by 0.5M NaCl, along with the increase in pH of the carrier solution. Absorption of phenolic compounds was apparently due to hydrophobic interaction between the compounds and the uncapped base material of the resin. Cellulose base resins, such as DEAE-sepharose (Hasegawa et al., 1989), have less affinity for the phenolics.

This system did not completely separate phenolics from limonoid glucosides. However, it provided a highly concentrated limonoid glucoside fraction up to 200 fold purification (Table 6). The system, using existing debittering facilities, could be expanded to industrial scale with less initial cost, at processing plants where the debittering system is functional.

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Lemon Juice Aroma Concentration by Reverse Osmosis

L. KANE, R.J. BRADDOCK, C.A. SIMS, and R.F. MATTHEWS

ABSTRACT

Ethanol, a major component of lemon juice, was analyzed to indicate degree of lemon juice aroma concentration. Aroma solution was concentrated from 12.2 to 23.4% ethanol at 8.3 MPa and 19°C by reverse osmosis. Ethanol rejected by the membrane decreased from the initial 87.7% to 78% at the final aroma concentration of 23.4% ethanol. Membrane rejection of neral, geranial, and terpinene-4-ol was > 96% while rejection of the alcohols, n-propanol, 2-methyl-3-buten-2-ol, and 3-methyl-2-buten-1-ol, ranged from 6.9% to 83.5%. Sensory analysis indicated no difference between initial aroma and aroma concentrated by reverse osmosis. Mass balance determined the true recovery of total volatiles was 79.7%. Loss of volatiles was attributed to adsorption in the polymeric matrix of the membrane and some loss was attributed to cleaning.

Key Words: reverse osmosis, aroma, neral, geranial, lemon juice

INTRODUCTION

TRADITIONAL METHODS to make processed citrus juices involve evaporation to concentrate the juice, which may require high temperatures to evaporate the water. The volatile constituents are removed with the water vapor and are lost from the concentrate, resulting in loss of fresh juice aroma. Aroma solutions are usually collected from juice during the initial stages of evaporation (Johnson and Vora, 1983) and may be added back to the final product.

Reverse osmosis (RO) has potential to concentrate aroma from citrus juices and is a nonthermal process. RO is a crossflow filtration process in which components of a fluid are separated by polymeric semi-permeable membranes by applied pressure (Paulson et al., 1985). Sheu and Wiley (1983) concentrated 10°Brix apple juice (at 25°C) to 20-25°Brix with high retention of volatiles and showed that lower operating temperatures gave better recovery of volatiles. Experiments with wine showed no loss of color with membrane concentration at 14.3% ethanol (Bui et al., 1988). Matsuura et al. (1974) evaluated concentration of apple juice and showed that, as concentration increased, the osmotic pressure of the juice increased and the flux, or production of permeate, decreased. Bowden and Isaacs (1989) reported permeation rate was affected by temperature, pressure, membrane type, and concentration. Matsuura et al. (1974) concluded from experiments with apple juice aromas that reverse osmosis yielded best results when performed in more than one stage. The purpose of the first stage was primarily concentration of aroma; while in the second stage, volatiles were recovered from the permeate. The same researchers later optimized aroma recovery by increasing pressure and lowering the processng temperature during the second stage (Matsuura et al., 1975).

Quality of citrus juices is determined mostly by the fresh juice-like characteristics of the aroma; therefore, retention of such components would be crucial for successful application of any alternative concentration process. Braddock et al. (1991) followed two small molecular weight volatiles, ethanol and acetaldehyde, in the reverse osmosis concentration process of orange essence. They concluded that rejection was improved at decreased temperature and increased pressure. Higher alcohol concentration resulted in decreased effectiveness of the membrane to reject ethanol and acetaldehyde. Higher than 50% rejection of ethanol and acetaldehyde was found when the concentration of alcohol in the process stream was < 10–25% ethanol. Greater rejection occurred with larger molecules than smaller ones. Based on the success of that study with orange aroma, the potential for successful concentration of lemon aroma by reverse osmosis was promising.

Lemon aroma solution is a value-added by-product of the evaporative concentration of lemon juice. It contains many compounds known to be sensitive to thermal degradation and oxidative conditions (Shaw, 1977). Lemon oil contains two to five major volatiles that are important contributors to its characteristic flavor; whereas, flavors in orange and mandarin are a result of mixtures of numerous components (Shaw, 1991) with many primary contributors. The quality of lemon oil is primarily dependent on the quantity of citral, the mixture of two isomers, neral and geranial (Shaw, 1979; Ikeda et al., 1962). Recovery of these volatiles would be required to maintain a high quality product.

Our objective was to investigate the effectiveness of reverse osmosis to concentrate lemon aroma.

MATERIALS & METHODS

Chemicals

All compounds used for quantitative analysis were purchased from Aldrich Chemical Company (Milwaukee, WI); chemicals and purity by gas chromatorgraphy are as follows: acetone 99%, n-propanol 99%, 2-methyl-3-buten-2-ol 98%, isobutanol 99%, butanol 99%, isoamyl alcohol 99%, trans-2-hexenal 99%, cis-3-hexen-1-ol 98%, octanal 99%, 1,8 cineole 99%, octanol 93%, 3-methyl-2-buten-1-ol 99%, linalool 95%, terpinene-4-ol 96%, nerol 97%, citral 80%, α-terpineol 98%, and geraniol 98%. Butyl acetate (95% purity by GC), methanol (optima grade), ethanol (HPLC grade), PrepSep™C_{18} resin disposable extraction columns, and citric acid were purchased from Fisher Scientific (Fairlawn, NJ). Lemon aroma (#1331) used as feed solution was a gift from Firmenich (Safety Harbor, FL).

Analysis

Lemon aroma was analyzed using GC. Analysis was preformed with a Hewlett-Packard 5890 GC equipped with a flame ionization detector (FID) connected to a 3396A integrator (Hewlett Packard Co., Avondale, PA). Separation was performed using a 0.53 mm × 30m DB5 (megabore) fused silica capillary column purchased from J&W Scientific Co. (Folsom, CA). Samples (0.5 µL) were injected into the GC at the following conditions: split ratio 6:1, H_2 carrier = 4.63 mL/min, injection port = 250°C, oven = 50°C (3 min) increased 6°C/min to 200°C (5 min), detector = 250°C. Triplicate injections were made of each sample. Analysis of chromatograms was performed on Quattro Pro (Borland International, Inc., Scotts Valley, CA) and Chromperfect 4.0 (Justice Innovations, Palo Alto, CA). Standard solutions were prepared from 10 to 300 ppm in 12% ethanol. Butyl acetate (100 ppm) was added as internal standard.

Identification of volatiles in the aroma (Moshonas and Shaw, 1972) was confirmed by mass spectroscopy using a Hewlett Packard 5971A MSD Mass Spectrometer (Hewlett Packard, Atlanta, GA) equipped with a 0.25 mm × 60 m DB5MS column from J&W Scientific (Folsom, CA). Aroma (1 µL) was injected into the GC/MS with operating conditions: H_2 carrier = 0.93 mL/min, split ratio 5:1, injection port = 250°C, inlet

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Table 1—Identification of volatiles in lemon aroma by gas chromatography confirmed by mass spectroscopy

methanol[a,b]	octanal
ethanol[a,b]	limonene
acetone	1-8-cineole
n-propanol[a]	octanol
2-methyl-3-buten-2-ol[a]	cis linalool oxide
isobutanol[a]	linalool[a]
butanol[a]	fenchyl alcohol
1-penten-3-ol	terpinene-4-ol[a]
1-1-diethoxyethane	α-terpineol[a]
isoamyl alcohol[a]	nerol[a]
3-methyl-2-buten-1-ol[a]	geraniol[a]
trans-2-hexenal	neral[a]
cis-3-hexen-1-ol	geranial[a]

[a] Volatiles monitored in reverse osmosis experimentation.
[b] Methanol and ethanol were not included in calculations of % rejection of total volatiles. They were analyzed separately.

of mass spectrometer = 270°C, oven = 50°C (3 min) increased 6°C/min to 200°C (0.50 min). Spectral analysis was performed using the Chemstation computer program supplied with the Wiley library (Hewlett Packard Software, Atlanta, GA).

Trapping volatiles

Permeate streams, as well as any aroma to be analyzed by mass spectroscopy, were concentrated to increase accuracy. PrepSep™C_{18} resin disposable extraction columns (Fisher Scientific Co., Fairlawn, NJ) were conditioned by eluting methanol and water. Aroma (10 mL) was then passed through the adsorbent, followed by rinse water, and eluted with 0.5 mL of methanol. Percent recovery of volatiles and level of concentration were determined by trapping volatiles from a solution (10 mL) containing 40 ppm of each of the volatiles and then eluting them with 0.5 mL methanol. The volatiles were concentrated 28-fold. Concentration of volatiles in the permeate stream from membrane concentration was calculated as follows: (average peak area/recovery factor for volatile)/28.

Reverse osmosis: Concentration of lemon aroma

Reverse osmosis experiments were performed using a Model 6410 Permasep B-9 permeator containing a polyamide-type hollow fine fiber membrane (SeparaSystems, Lakeland, FL). The membrane was cleaned by continuous recycling with 0.5% Belle Enz-HPH detergent (Belle Corp., Burlington, IA) at pH 12 and post-treated with an 80 mg/L tannic acid and 1% citric acid solution as suggested by the manufacturer. Rejection of a 2% salt (NaCl) solution was 98%. Initial experiments were performed at various temperatures (13°C, 19°C, and 25°C) and pressures (5.5 MPa, 6.9 MPa, and 8.3 MPa) to determine effects of the process on aroma solutions as well as optimum operating conditions.

After initial expermients, aroma solution (12 L) was concentrated from 12.2 to 23.4% ethanol at the optimum conditions of 8.3 MPa and 19°C. Samples of feed and permeate were taken and the permeation rate was measured. Two replications (Trials 1 and 2) were performed. All samples were purged with nitrogen gas and held at 4°C until analyzed. Feed and concentrate streams were analyzed by direct injection into the GC. Permeate streams were concentrated using the adsorbent and then injected into the GC. Percent rejection was calculated as 1 - ([permeate]/[feed]) × 100%. In this analysis, the term "percent rejection" was used to describe the percent of materials being rejected by the membrane with respect to the initial concentration of the feed and permeate (Podall, 1972). The surface area was not disclosed by the manufacturer; therefore, permeation rate was expressed as L/hr instead of L/hr/m^2. The surface area for this type of membrane was reported to be about 50 m^2 (Caracciolo et al., 1974).

Sensory analysis

Aroma samples (4 L) were diluted with distilled water (12 L) then concentrated to original strength by reverse osmosis at 8.3 MPa and 19°C. Samples of feed and concentrate streams were taken (Trial 1) and this experiment was replicated (Trial 2). A tasting formula for sensory analysis consisted of aroma diluted to 0.15% ethanol, 0.2% citric acid, and 8°Brix (Hendrix, 1993). Brix was measured using a Reichert ABBE Mark II digital refractometer from AO Scientific Instruments (Buffalo, NY). Triangle tests were performed comparing the unprocessed and concentrated aroma from Trial 1 given to 24 panelists. Triangle tests were repeated using the concentrated aroma from Trial 2 and given to 23 panelists. Results of both tests were combined, totaling 47 panelists. All samples were served at room temperature. Analysis of the sensory results was by comparison charts (Roessler et al., 1948).

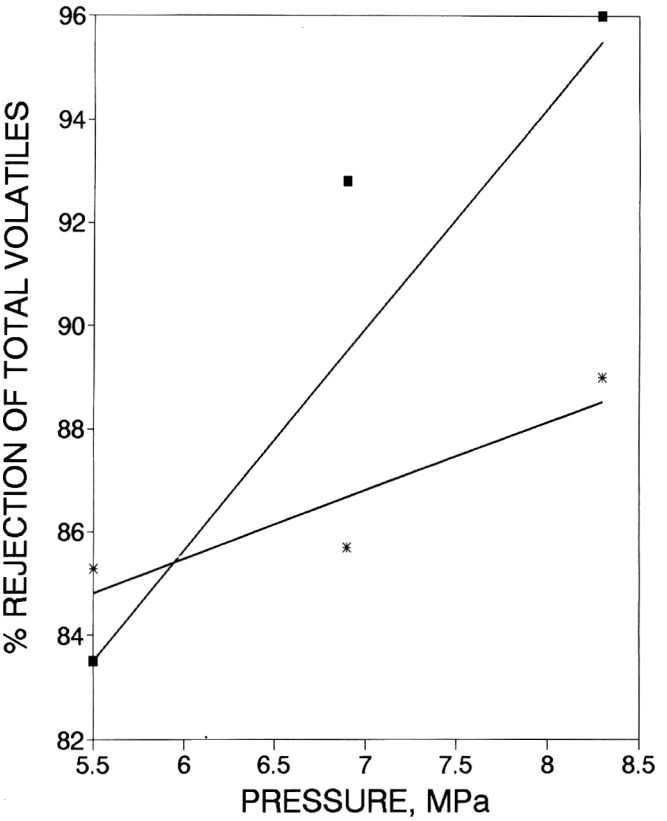


Fig. 1—Rejection of total volatiles at (■ - ■) 13°C (n = 2, R^2 = 0.93, Std Error = 2.49) and (* - *) 25°C (n = 2, R^2 = 0.92, Std Error = 1.19) at 5.5, 6.9, and 8.3 MPa.

RESULTS & DISCUSSION

Lemon aroma compounds

Mass spectroscopy helped identify 26 compounds in the lemon aroma (Table 1). Major constituents were α-terpineol and terpinene-4-ol as well as neral, geranial, linalool, and 2-methyl-3-buten-2-ol. Many of these volatiles had been also identified by Moshonas and Shaw (1972). α-Terpineol and terpinene-4-ol can vary in concentration between lemon aroma samples because they are products of the hydration of limonene (Shaw, 1979). The volatiles in greatest concentration were selected to investigate recovery in the reverse osmosis process (designated in Table 1).

Initial analysis of pressure and temperature

Comparison of the two processing temperatures (Fig. 1) indicated total retention of volatiles was maximum at 8.3 MPa and 13°C. At 13 and 25°C, retention of total volatiles was 96 and 89%, respectively, at 8.3 MPa. At the higher temperatures, retention of volatiles decreased. Matsuura et al. (1975) also reported that lower temperatures and higher pressures yielded the best recovery. Losses of α-terpineol and terpinene-4-ol were more notable at 5.5 MPa and 6.9 MPa. n-Propanol, 2-methyl-3-buten-2-ol, and 3 methyl-2-buten-1-ol as well as α-terpineol and terpinene-4-ol were found in permeate streams at all pressures at 25°C. Temperature also affected permeation rate (Fig. 2).

Higher pressures increase the flow of water through the membrane which dilutes the permeate stream, therefore, increasing percent rejection of the retained volatiles (Goettsch, 1993; Po-

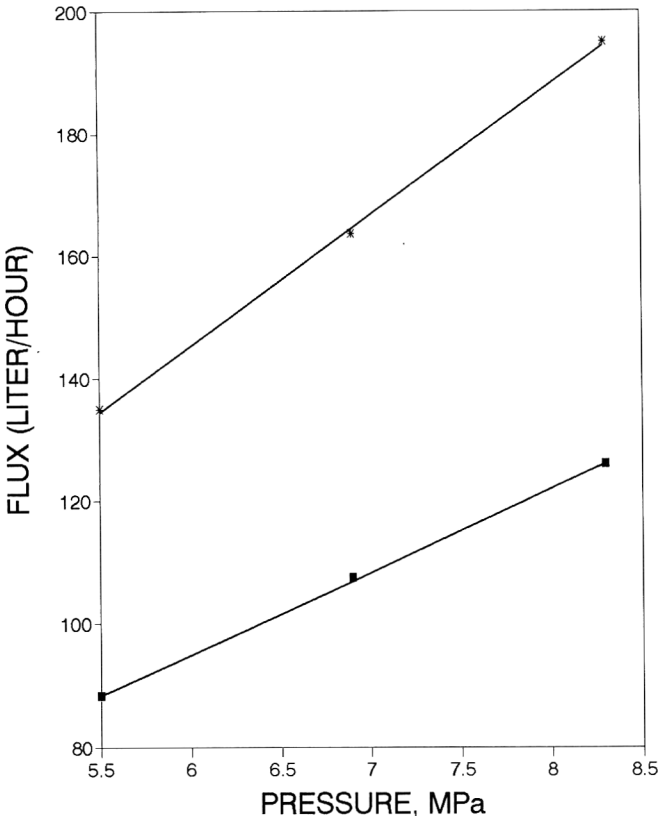


Fig. 2—Permeation rate (flux) at (■ - ■) 13°C (n = 2, R^2 = 0.99, Std Error = 0.004) and (* - *) 25°C (n = 2, R^2 = 0.99, Std Error = 0.024) at 5.5, 6.9, and 8.3 MPa.

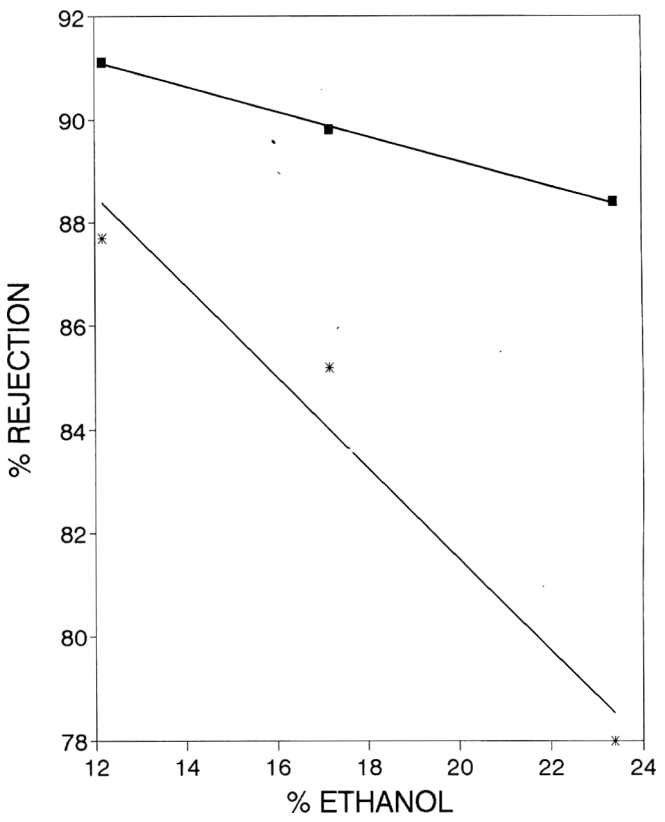


Fig. 4—Average % rejection of (■ - ■) total volatiles (n = 2, R^2 = 0.99, Std Error = 0.08) and (* - *) ethanol (n = 2, R^2 = 0.96, Std Error = 1.49) in concentrated lemon aroma at 8.3 MPa and 19°C with increasing % ethanol.

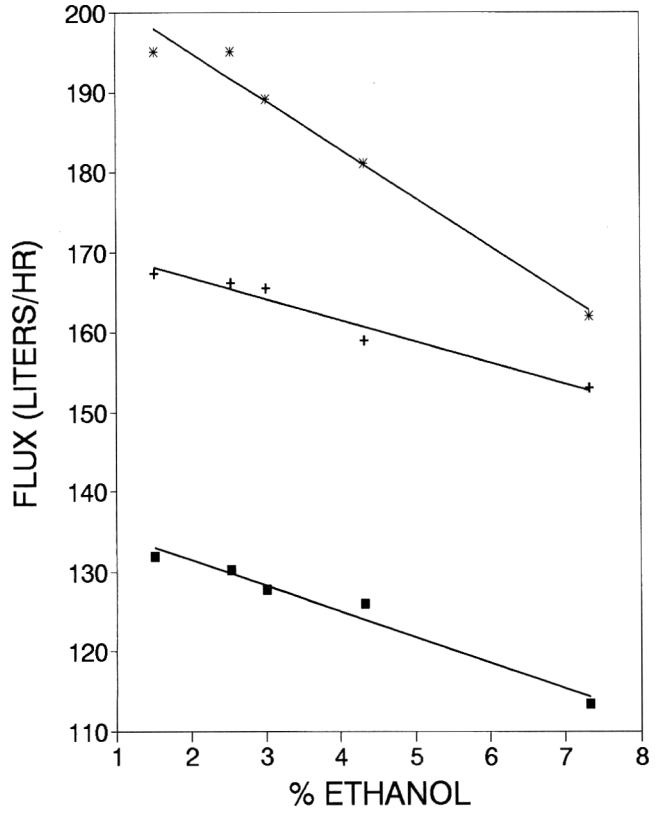


Fig. 3—Permation rate (flux) at 8.3 MPa at (■ - ■) 13°C (n = 2, R^2 = 0.97, Std Error = 0.025), (+ - +) 19°C (n = 2, R^2 = 0.96, Std Error = 0.01), and (* - *) 25°C (n = 2, R^2 = 0.97, Std Error = 0.043) at ethanol concentrations between 1 and 8%.

dall, 1972). The most efficient conditions for concentration were determined to be 8.3 MPa and 25°C with a permeation rate of 195 L/hr. This was 54.6% faster than the permeation rate at 13°C. Sheu and Wiley (1983) also reported that increasing temperature and pressure increased permeation rate (Fig. 3).

Initial concentration experiments indicated that retention of the less volatile oxygenated terpenes, as well as aldehydes important to lemon flavor (neral, and geranial) were > 90% and were unaffected by temperature. The lower molecular weight, more volatile alcohols such as n-propanol and 2-methyl-3-buten-2-ol were more affected by increase in temperature with average recoveries from 15 to 90%. In aroma with 12.2% ethanol, rejection of ethanol at 19°C was 89% (Fig. 4). These values confirmed those reported by Bui et al. (1988) who increased the concentration of alcohol in wine from 11.5 to 12.5% ethanol, noting an 85% rejection of ethanol.

Concentration of lemon aroma

In preliminary experiments, the best permeation rate was found at 25°C and the best rejection values were at 13°C. Therefore, concentration of the lemon aroma was at 19°C and 8.3 MPa to give good recovery of volatiles as well as a permeation rate with good efficiency. With the pressure experiments, 8.3 MPa resulted in highest rejection; therefore, all remaining concentration experiments were performed at that pressure. Aroma was concentrated from 12.2 to 23.4% ethanol. Processing streams of the concentration experiment (Table 2) showed lower total volatile percent rejection at higher alcohol concentration. The reason is unclear but some volatile compounds would have greater solubility at higher alcohol concentrations and be less likely to remain in the retentate.

Analysis of the individual compounds (Table 3) showed most terpene compounds were only slightly affected by the increase in ethanol concentration of the aroma concentrates with rejec-

Table 2—Concentration of aroma compounds in process streams

	Feed[a] (mg/L)		Concentrate[b] (mg/L)		Composite permeate[c] (mg/L)	
n-propanol	53.6	(5.0)[d]	131.4	(2.5)	97.6	(4.2)
2-methyl-3-buten-2-ol	178.0	(17.1)	520.4	(26.0)	63.9	(4.8)
isobutanol	15.5	(1.3)	32.8	(5.4)	18.0	(0.5)
butanol	11.8	(0.5)	20.9	(0.3)	9.1	(1.5)
isoamyl alcohol	20.2	(1.4)	47.1	(0.6)	7.6	(1.1)
3-methyl-2-buten-1-ol	27.7	(2.2)	67.1	(1.5)	37.8	(4.0)
linalool	20.8	(1.2)	49.5	(0.5)	0.3	(0.0)
terpinene-4-ol	158.2	(13.6)	434.4	(2.5)	7.5	(3.0)
α-terpineol	269.3	(24.5)	750.4	(6.1)	13.8	(4.5)
nerol	12.6	(0.9)	27.4	(0.2)	0.0	(0.0)
geranicl	13.5	(0.6)	27.1	(0.2)	0.0	(0.0)
neral	18.2	(0.7)	45.9	(0.6)	0.5	(0.4)
geranial	15.1	(0.1)	33.4	(0.5)	1.1	(0.7)

[a] Initial feed stream as 12.2% ethanol.
[b] Concentrate stream was 23.4% ethanol.
[c] Composite permeate stream was 5.4% ethanol.
[d] Standard error expressed as percent in parentheses.

Table 3—Percent rejection of volatile compounds at 8.3 MPa and 19°C at aroma concentrations of 12.2, 17.2, and 23.4% ethanol (pooled standard error of means for the % rejection of two trials at all concentrations = 13%)

	Membrane rejection (%)		
	Percent ethanol		
Volatile	12.2%	17.2%	23.4%
n-propanol	38.7	25.8	6.9
2-methyl-3-buten-2-ol	88.5	87.7	82.6
isobutanol	60.2	52.2	40.5
butanol	70.9	56.5	52.0
isoamyl alcohol	74.5	83.9	75.9
3-methyl-2-buten-1-ol	47.3	43.8	28.3
linalool	99.6	99.7	99.7
terpinene-4-ol	95.9	98.3	97.8
α-terpineol	97.0	98.2	97.6
nerol	100.0	100.0	100.0
geraniol	100.0	100.0	100.0
neral	99.0	98.9	98.6
geranial	97.6	96.7	96.2

Table 4—Recovery of volatile compounds in lemon aroma concentrated by reverse osmosis

Volatile	Average recovery (%) ± std dev n = 2
n-propanol	77.6 ± 14.8
2-methyl-3-buten-2-ol	70.8 ± 17.8
isobutanol	78.3 ± 13.6
butanol	90.1 ± 6.5
isoamyl alcohol	85.9 ± 10.9
3-methyl-2-buten-1-ol	78.8 ± 13.2
linalool	77.7 ± 8.7
terpinene-4-ol	70.9 ± 14.6
α-terpineol	90.8 ± 10.8
nerol	70.5 ± 14.9
geraniol	85.5 ± 8.0
neral	75.8 ± 4.1
geranial	83.4 ± 4.2

tions > 96%. For example, the important lemon flavor compounds, geranial and neral, were rejected between 96 to 99% with increasing concentrations of ethanol. More notable changes in rejection were demonstrated with some of the smaller molecule alcohols. For example, n-propanol, 3-methyl-2-buten-1-ol, isobutanol, butanol, and isoamyl alcohol were all affected by an increase in ethanol concentration indicated by a decrease in rejection. n-Propanol had the lowest rejection at 38.7% at 12.2% ethanol and decreased to 6.9% at 23.4% ethanol. n-Propanol is a small molecular weight compound capable of penetrating the membrane. 2-Methyl-3-buten-2-ol had higher rejections at all ethanol concentrations than did 3-methyl-2-buten-1-ol.

The percent rejection of ethanol (Fig. 4) decreased as ethanol concentration increased. Sheu and Wiley (1983) found the rejection of ethanol was greater than 70% when concentrating apple juice flavors. Braddock et al. (1991) reported rejection of ethanol was 60% at 20% ethanol concentration. The permeation rate of the aroma decreased from 75.6 L/hr to 65.4 L/hr as the aroma was concentrated from 12.2 to 23.4% ethanol. This was significantly lower than the permeation rate of 153 L/hr calculated at 7.3% ethanol. We could not compare the permation rates to published values because our membrane surface area was not known. The membrane used had an exceptionally high surface area in a very small volume.

Determination of the true recovery of volatiles from reverse osmosis concentration was achieved by comparing the initial aroma to the aroma concentrated to 23.4% ethanol, both diluted to 6% ethanol. The recoveries of the volatile compounds were compared (Table 4). Average recovery of volatiles was 79.7%. α-Terpineol had the greatest recovery of 90.8%. Recoveries of neral and geranial were 75.8 and 83.4%, respectively.

The original aroma was compared to concentrated aroma in this manner to indicate the amount of each compound lost during the process. Losses may occur by either adsorption into the polymeric matrix of the membrane or during the cleaning process. Recovery could probably be increased by concentrating the rinse water used to clean the membrane and adding those volatiles back to the concentrated aroma. Recovery of volatiles on an industrial scale would be higher than those reported because the concentration process would be continuous on a larger unit. Also, cleaning may not be as frequent, and the amount of volatiles bound to the membrane would represent a smaller fraction of total volatiles.

Sensory analysis

The final concentration experiment was performed to determine if the loss of any of the flavors was detectable by taste panel. Of 47 panelists participating in triangle tests, only 10 detected a difference. On the survey, most panelists commented that difference between the samples was slight, if at all detectable. For this analysis, we concluded with 99.9% confidence that there was no detectable difference between samples. This could be due to the high recovery of neral and geranial, allowing efficient concentration and very little loss into the permeate. Neral and geranial are the main constituents responsible for flavor in lemon, and the other compounds enhanced or embellished the flavor (Shaw, 1991).

Because the rejection of neral and geranial was >98.6 and 96.2% respectively, we could deduce that the alcohol compounds that had greater losses such as n-propanol and 2-methyl-3-buten-2-ol had less impact on flavor.

CONCLUSIONS

HIGHER TEMPERATURES resulted in decreased rejection of volatiles with high permeation rates; while at lower temperatures, rejection increased and permeation rates decreased. The highest pressure resulted in highest rejection irrespective of temperature. As solute concentration increased, both permeation rate and volatile rejection decreased. Optimum concentration included using the highest pressure and a medium temperature for good rejection of volatiles, while maintaining reasonable permeation rates. Reverse osmosis has a good potential as a concentration process for lemon aroma. Because this process is nonthermal, the compounds necessary for maintaining high quality aroma are retained and not degraded.

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Spray Drying of *Lactococcus lactis* ssp. *lactis* C2 and Cellular Injury

WAN-YIN FU and MARK R. ETZEL

ABSTRACT

Starter cultures were spray-dried at five outlet-air temperatures using four concentrations of cells in the feed solution. Powders made using the lowest outlet-air temperature and the highest cell concentration had the highest viability. Storage at 4°C for 3 mo caused a 34–85% loss of viability. Cellular injury resulted from dehydration, and exposure to high temperatures in the atomizer and during droplet drying. Lactic acid production was similar for frozen, freeze-dried and spray-dried cultures made from a single cell paste. The lag time before lactic acid production was apparently an inherent characteristic of each specific cell paste.

Key Words: starter culture, lactic acid bacteria, spray drying, cellular injury

INTRODUCTION

LACTIC ACID BACTERIA are used as starter cultures in food fermentation processes for yogurt, cheese, and sausage. In addition, lactic acid bacteria (LAB) are used as probiotics and as silage inoculants. Starter cultures are commonly preserved by freezing or freeze drying (Champagne et al., 1991). Frozen cultures contain 90% water, are heavy and bulky, and require sub-zero temperaures for shipment and storage. Freeze-dried cultures are light and compact, but are costly to produce. Spray-dried cultures may have similar advantages to freeze-dried cultures, but would be much less costly to produce. However, because starter cultures are heat sensitive, special attention must be given to the design and operation of the spray dryer to provide viable cultures with rapid lactic acid production upon rehydration. This requires understanding the mechanisms by which cellular injury occurs during spray drying.

In most research on spray drying of lactic cultures, cell viability but not lactic acid production has been measured (Johnson and Etzel, 1993; Daemen and van der Stege, 1982; Kim and Bhowmik, 1990; Metwally et al., 1989). Cell viability, but not lactic acid production, is important for starter cultures used as probiotics and as silage inoculants. Lactic acid production, but not cell viability, is important for starter cultures used in making yogurt and cheese. Losses in cell viability due to thermal inactivation during spray drying has been modeled mathematically and compared qualitatively to starter culture viability after spray drying (Daemen, 1984; Fu et al., 1994). Decreases in drop size, outlet-air temperature, and solids content in the feed solution have decreased thermal inactivation. Losses in cell viability due to cell dehydration may also be important during spray drying of starter cultures (Lievense et al., 1992, 1994; Lievense and van't Riet, 1993a,b). Lactic acid production of spray-dried cells has been briefly reported for *Lactobacillus helveticus* (Johnson, 1993; Johnson and Etzel, 1994), *Streptococcus cremoris* and *Streptococcus lactis* (Foster, 1962). However, it was not the main emphasis of the reports.

Our objectives were to determine: (1) the effect of outlet-air temperature and cell loading in the feed solution on cell survival, lactic acid production and stability after spray drying, and (2) the location and mechanism for cellular injury during spray drying. Injury caused by atomization, heating inside the atomizer, and cell dehydration was measured by determining the survival and the lactic acid production from treated cells. Cultures were frozen, freeze dried or spray dried to determine effects of different processing methods on cellular injury. These results were used to study the mechanisms of cellular injury during spray drying.

The intent of our study was to elucidate the effects of spray drying on starter cultures, and to compare culture characteristics between cells grown similarly but processed by freezing, freeze drying or spray drying. We did not attempt to compare spray-dried cell suspensions to commercially prepared frozen or lyophilized cells. However, the results of this study may be useful in developing economical commercial processes for spray-dried starter cultures.

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MATERIALS & METHODS

Cell cultivation

A single strain of *Lactococcus lactis* ssp. *lactis* C2 (alias *Streptococcus lactis* C2) was provided by Prof. J.S. Steele, Department of Food Science, University of Wisconsin, Madison. It was streaked onto M17 agar plates, and four to six colonies used to inoculate 10 mL of M17 broth (Terzaghi and Sandine, 1975). All colonies were used for inoculation to avoid possible loss of plasmid from a single colony. After growth for 1 day at 32°C, 0.3 mL of culture solution was dispersed into 2 mL vials containing 1.0 mL of 11% nonfat dry milk and 0.3 mL of 50% glycerol. The vials were stored at −78°C.

For each set of experiments (Fig. 1), one vial of cells was thawed and 0.2 mL of culture solution used to inoculate 10 mL of M17 broth. After growth at 32°C for 19 hr, 2 mL of the culture were added to 100 mL of M17 broth and incubated at 32°C for 12 to 13 hr. A 1% inoculum of the culture was then added to M17 broth. The culture was grown at 32°C for 8 hr with constant stirring in a spinner flask (model 1969-03000 or 1969-08000, Bellco Glass, Inc., Vineland, NJ). The fermentation was maintained at pH 6.3 using a pH controller (model 45A, Chemtrix, Inc., Hillsboro, OR) and a neutralizing solution containing ammonium hydroxide and sodium carbonate. Cells were harvested in the early stationary phase by centrifugation (6000 × *g* for 15 min or 2500 × *g* for 50 min at 4°C). Acids and excess media in the cell paste were removed by resuspending the cells in cold 0.01*M* sodium phosphate buffer, pH 7, followed by centrifugation. The cell paste was stored at 4°C.

Preparation of feed solutions

From 37 fermentations, each cell paste was designated by a batch number. One-day-old cell paste from a single batch was dispersed in 0.01 *M* sodium phosphate buffer, pH 7, and stirred until homogeneous. The cell suspension was added to 30% solids condensed skim milk (University of Wisconsin-Madison Dairy Plant). Distilled water was added to provide a final total solids content of 25 ± 1.5%. The amount of cell paste used was 3, 30, 100 and 200 mg/mL of final feed solution yielding 0.3, 3, 10 and 20% cell loading, respectively. The colony-forming units (CFU) of the feed solutions were: 1.6(.4) × 10^9/mL (n = 8) for 0.3% cell loading; 1.5(.2) × 10^{10}/mL (n = 4) for 3% cell loading; 6.7(.5) × 10^{10}/mL (n = 2) for 10% cell loading; and 1.1(.2) × 10^{11}/mL (n = 9) for 20% cell loading [mean (S.D.); n = batches of cell paste].

Spray drying

A bench-top-scale spray dryer (model SD-04, Virtis Company, Inc., Gardiner, NY) was used (Fig. 2). Air was heated electrically to 220 ± 1°C after passing through a blower delivering 600g of air/min (0.47 m^3/min at STP). The feed solutions were held in an ice bath during spray

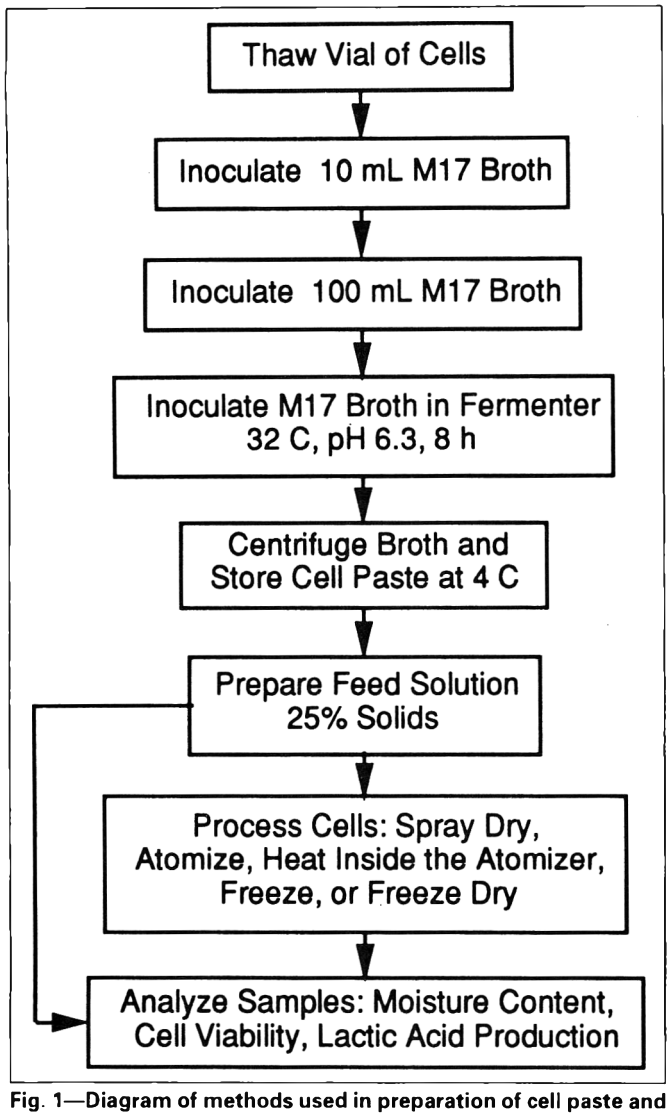


Fig. 1—Diagram of methods used in preparation of cell paste and feed solution.

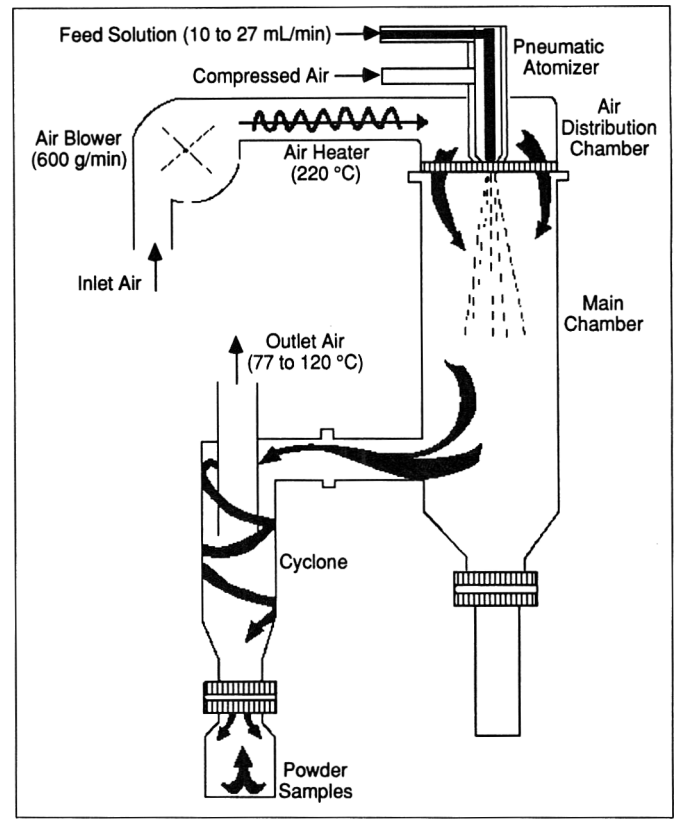


Fig. 2—Sketch of equipment and conditions for preparation of spray-dried cultures.

drying. A peristaltic pump (model 7524-10, Cole-Parmer Instr. Co., Chicago, IL) delivered the feed solutions to a two-fluid stainless-steel atomizer with a liquid jet with inside diameter 1 mm. Inlet air flowed cocurrently with the spray through the main chamber of the spray dryer which was 48.5 cm in length and had an inside diameter of 10.5 cm. Outlet-air temperatures from 77 to 120°C were controlled by adjusting the flow rate of the feed solution. Feed solution flow rates of 27, 23, 17, 13 and 10 mL/min provided outlet-air temperatures of 77, 90, 100, 110 and 120°C, respectively. Spray-dried powder samples were collected in sequence starting with low and ending with high outlet-air temperatures. Dried powder samples were collected from the base of the cyclone and mixed thoroughly with a spatula. The samples were stored in tightly sealed sterile bottles at 4°C.

Atomization

To measure the effect of atomization on cellular injury, (i.e. that due to shear forces), a feed solution with 3% cell loading was sprayed into a sterile beaker held directly under the atomizer. The electrical heat for the inlet air was turned off. Flow rate of the feed solution was 27 mL/min. The sample was immediately transferred to a capped bottle and stored on ice prior to analysis.

Heating inside the atomizer

The atomizer was located at the center of the inlet-air distribution chamber in which the air was at 220°C (Fig.2). Cells could be injured while traveling through the hot atomizer. To measure the extent of any cellular injury from this, a feed solution with 3% cell loading was pumped into the atomizer at 27 mL/min. The dead volume in the atomizer was 2.6 mL, from which the calculated residence time of the cells in the hot atomizer was 0.1 min. With compressed air off, the electrical heater for the inlet air was turned on allowing the inlet-air distribution chamber to reach 220°C. Thick-walled silicone rubber tubing was connected to the outlet of the atomizer. This shielded the feed solution from the 220°C air emerging from the inlet-air distribution plate such that only cellular injury due to heating inside the atomizer would be measured. Samples of heated (but not atomized) feed solution were collected from the tubing in sterile bottles and kept in ice water until analysis.

Freezing and freeze drying

To measure the extent of cellular injury due to dehydration, feed solutions with 3% cell loading were frozen and freeze dried (Bench Top 3, The Virtis Company, Inc., Gardiner, NY). Round bottom flasks (100 mL) were filled with either 80 mL (no. 34, Table 3) or 50 mL (no. 35 and 37) of feed solution and frozen by rotating the flasks in a 95% ethyl alcohol bath (−40 to −50°C) for 25 min. Six flasks were prepared for each batch. Half the flasks were stored at −20°C for 2 days, while the others were connected to the freeze dryer (pressure 27 Pa and temperature −52°C) and dried for 2 days.

Moisture content

Moisture content was computed based on weight lost after drying. Moisture contents of cell pastes, feed solutions, and rehydrated powder samples were determined by placing 1 mL in an aluminum weighing dish and drying in a convection oven at 70°C for 14 hr. For such low moisture contents as the powder samples had, moisture gain from the air could be considerable. Therefore, 1 g of each spray-dried powder sample was dried and then covered with an aluminum dish immediately after drying. Moisture contents of all samples were calculated from triplicate analyses except duplicates were used for the cell pastes.

Cell viability

Cell viability of the paste, feed solutions and rehydrated samples was determined using the standard plate count method (Messer et al., 1985). Spray-dried powders were rehydrated with sterile deionized water to

yield about the same solids concentrations as the feed solutions. Rehydration with water instead of milk facilitated comparing plate counts of rehydrated samples and feed solutions. The rehydrated samples were mixed periodically for 2 hr while immersed in ice water. The time required indicates the powder was difficult to dissolve. Keeping the samples in ice water prevented cell growth during dissolution. The samples were plated onto M17 agar (Difco Laboratories, Detroit, MI). Colony-forming units were counted after incubation at 32°C for 5 days.

Values reported for CFU/mL of the cells after processing or after drying and rehydration were corrected to have the same solids concentrations as feed solutions. The correction was made using the equation: $N = N_m \times S_f/S_m$, where N was the value reported for the CFU/mL of a rehydrated solution with the same solids concentration as the feed solution, N_m was the CFU/mL of the measured solution, S_f was the solids concentration of the feed solution, and S_m was the solids concentration of the measured solution. Percent survival was reported as 100 (N/N_o), where N_o was the CFU/mL of the feed solution.

Lactic acid production

Samples of spray-dried powder, cell paste, feed solution or frozen cells were transferred aseptically to a milk dilution bottle and diluted to 100 mL with pasteurized 9% solids reconstituted non-fat dried milk. The amount of each sample was adjusted to yield a constant final total cell concentration (injured and uninjured) in all assays equivalent to 11 mg of cell paste per 100 mL milk solution (Fu, 1994). This was done to standardize the number of cells in each dilution bottle before measurement of lactic acid production, because each sample of cells initially had very different CFU/mL. Thus, 1g of cell paste was reconstituted in 99 mL of 0.01M sodium phosphate buffer, pH 7. Then, 1.1 mL of the solution was diluted to 100 mL with milk solution prior to measurement of lactic acid production. The calculated viable cell count was $\approx 5 \times 10^7$ CFU/mL in bottles containing cell paste and feed solution, and was proportionately lower in those containing cells with a lower percentage of survival due to inactivation of a portion of the cells.

The milk solutions were held at 32°C in a water bath. Lactic acid production was determined by removing 9-mL aliquots periodically over 0 to 25 hr and measuring pH. Milk solution without inoculum served as control.

RESULTS & DISCUSSION

Moisture content of the spray-dried powder

Moisture contents of the spray-dried powders ranged from 5.8% to 0.2% (Table 1). Moisture content did not depend on cell loading in the feed solution. However, moisture content decreased as outlet-air temperature increasd from 77 to 120°C. As described earlier, the flow rate of the feed solution was decreased to increase the outlet-air temperature. Decreasing the liquid flow rate increased the air temperature and decreased relative humidity. These changes resulted in more rapid drying. Increased drying rates probably caused the decrease in moisture content with increased outlet-air temperature. This result was observed both experimentally and theoretically by Johnson and Etzel (1993), and Etzel et al. (1994) for spray drying of milk solutions.

Cell survival after spray drying

For all levels of cell loading, increasing outlet-air temperature decreased percent cell survival (Table 2). This has been found for other spray-dried LAB. This trend has been well documented for *Lactobacillus helveticus* (Johnson, 1993; Johnson and Etzel, 1993, 1994), for *Streptococcus lactis, Streptococcus thermophilus,* and *Lactobacillus bulgaricus* (Metwally et al., 1989; Kim and Bhowmik, 1990) and for *Lactobacillus acidophilus* (Espina and Packard, 1979). Other bacteria which followed this trend, were *Serratia marcescens* and *Staphylococcus* C131 (Daemen and van der Stege, 1982).

Increasing cell loading marginally increased percent survival after spray drying. However, the effect of cell loading was weak and within the variability of the data. The influence of cell loading was insignificant at the highest outlet-air temperatures.

Table 1—Moisture content of spray-dried powder samples as related to outlet-air temperature and cell loading in the feed solution

Outlet-air temp (°C)	% Cell loading in the feed solution			
	0.3	3	10	20
77	4.9[a] (0.2)[b]	4.3 (0.7)	4.3 (0.1)	5.8 (0.6)
90	3.2 (0.3)	2.4	2.6	3.7 (0.0)
100	1.8 (0.2)	1.6	1.5	2.7
110	1.2 (0.0)	0.6	0.5	1.0
120	1.2 (0.2)	0.5	0.2	0.7

[a] Moisture content (%).
[b] Mean (standard deviation) for replicate experiments. Means without S.D. are for triplicate analyses of single experiments.

Table 2—Changes in survival of all spray dried cell powders during storage at 4°C

% Cell loading	Days at 4°C	Outlet-air temperature (°C)				
		77	90	100	110	120
0.3	1	53[a] (8)[b]	40 (16)	26 (14)	10 (6)	1.3 (0.7)
	4	49 (5)	32 (7)	18 (10)	6 (4)	0.8 (0.6)
	32	32 (2)	27 (12)	17 (13)	5 (4)	0.6 (0.6)
	55	33 (10)	34 (21)	18 (14)	6 (5)	0.8 (0.8)
	91	25 (7)	27 (16)	18 (14)	6 (5)	0.5 (0.5)
3	1	61 (9)	36	20	5	0.7
	4	53	30	17	4	0.3
	32	35	22	11	2	0.1
	55	43	20	11	2	0.1
	91	25	17	8	1	0.1
10	1	59 (4)	51	29	9	1.1
	8	53 (4)	44	25	9	0.7
	32	48 (8)	34	16	5	0.4
	55	48 (11)	35	21	4	0.3
	91	39 (8)	24	16	4	0.3
20	1	78 (12)	59 (6)	36 (8)	11 (0)	1.6 (0.3)
	8	58	54	32	8	1.4
	32	54 (2)	50 (1)	25 (9)	5	0.7
	55	49	53	32	5	0.9
	91	36	50	24	4	0.5

[a] Percent survival.
[b] Mean (standard deviation) for replicate experiments. Means without S.D. are for replicate plate counts of single experiments.

The effect of cell loading on percent survival after spray drying has not been reported previously. For freeze drying, Kilara and Shahani (1976) found that increasing cell loading increased percent survival for four different LAB: *L. bulgaricus, L. acidophilus, S. thermophilus,* and *S. lactis.* Bozoglu et al. (1987) verified these findings for *L. bulgaricus* and *S. thermophilus,* and attributed the effect to a mutual shielding of microorganisms against severe conditions of the external media.

Increasing cell loading in the feed solution increased the throughput of cells from the spray dryer without decreasing cell survival. In addition, increasing the flow rate of the feed solution to the spray dryer decreased the outlet-air temperature, which increased both the utilization of the energy in the inlet air and increased cell survival. Based on these results such methods for increasing throughput may provide the additional benefit of increasing cell survival.

Cell survival during storage

Cell survival vs time was measured during 3 mo of storage at 4°C (Table 2). This temperature was used because it would be less costly to maintain than sub-zero temperatures used for commercially available frozen cultures. All samples had a gradual decrease in cell survival during storage.

The relative survival was calculated as percent cell survival during storage divided by percent cell survival at 1 day (from Table 2). Generally, as outlet-air temperature increased there was a more rapid decrease in relative survival during storage. The greatest decrease in relative survival occurred for cells spray-dried at the highest outlet-air temperature (120°C).

Two factors may have combined to cause those cells to have the greatest decrease in relative survival during storage. They

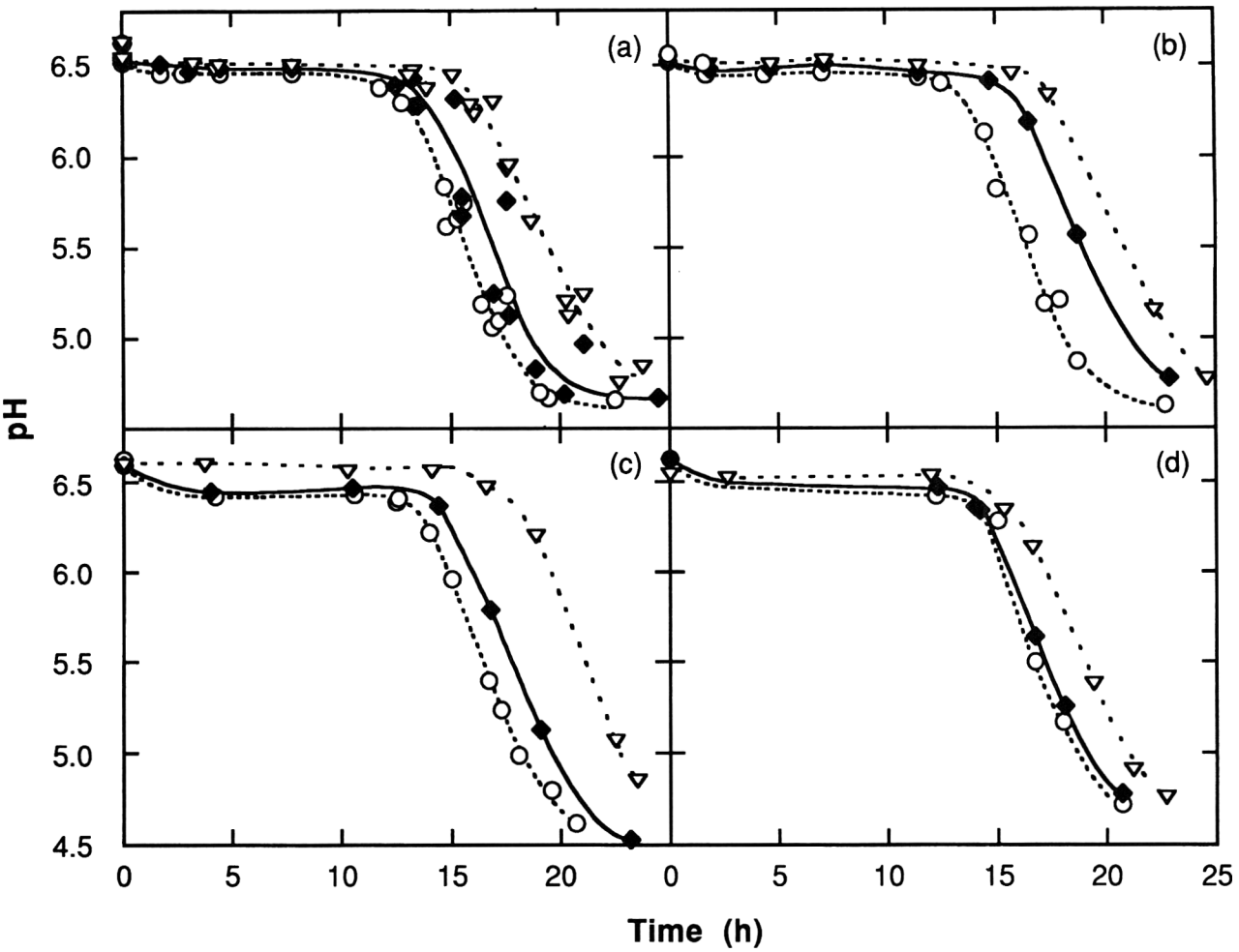


Fig. 3—Effect of outlet-air temperature and cell loading in the feed solution on the drop in pH due to lactic acid production of the cells rehydrated in 9% nonfat dry milk solutions at 32°C: (a) 0.3% cell loading; (b) 3% cell loading; (c) 10% cell loading; (d) 20% cell loading. (---○---) 77°C; (——◆——) 100°C; and (- -▽- -) 120°C.

had the greatest extent of unrepairable injuries, with 98 to 99% cell death after spray drying. Also, these cells had the lowest moisture contents of 0.2 to 1.2%. As reported by Champagne et al. (1991), removing that much water may expose cracks and void spaces in the dried particles to air, causing oxidative degradation of proteins and cellular compounds. Based on our results, cell survival during storage could be increased by spray drying at low outlet-air temperatures, controlling the powder moisture to 1 to 4%, and storing at refrigerated temperatures.

Lactic acid production of spray-dried cells

The drop in pH due to lactic acid production for spray dried *Lc. lactis* C2 was measured for four levels of cell loading in the feed solution, and three outlet-air temperatures (Fig. 3). Control samples consisted of un-inoculated milk solution, and did not change in pH until after 20 to 25 hr (data not shown). The lag time before lactic acid production was generally 12 to 16 hr, and increased slightly with increasing spray dryer outlet-air temperature. The rate of lactic acid production after the lag time was equivalent for all samples as evidenced by equivalent slopes of pH vs time curves after lag time. Cell loading did not significantly affect lactic acid production.

Lag times of 12 to 16 hr would not be practical in the production of cheese and yogurt where rapid lactic acid production is of great importance. However, for starter cultures used as probiotics and as silage inoculants, long lag times would not be a problem. Long lag times were not always observed for spray-dried starter cultures. When no lag times were observed for the cell paste, feed solution, and frozen cells, then the spray-dried starter cultures also had no lag times.

Atomization

Atomization of the feed solution during spray drying could cause cellular injury due to shear forces. However, when feed solution was subjected to atomization, all cells survived (Table 3). Either *Lc. lactis* C2 was not injured by atomization or possibly was broken into smaller chains, thus obscuring injury caused by atomization. The atomized cells were grown in 9% nonfat dry milk solution at 32°C, and lactic acid production was identical to that of the feed solution (Fu, 1994). Therefore, atomization alone did not appear to cause cellular injury. Similarly, Johnson and Etzel (1993) reported that after atomization *L. helveticus* survival ranged from 83% to 103%.

Heating inside the atomizer

Because the atomizer was located at the center of the inlet-air distributor, cells may have been injured while traversing the hot atomizer. The mean survival was 86% after the cells were heated in the atomizer (Table 3) but not atomized. Therefore, heating in the atomizer was a notable contributor to the overall effects of spray drying on cellular injury. However, when heated cells were grown in 9% non-fat dry milk solution at 32°C, lactic acid production was identical to that of the feed solution (Fu, 1994).

Table 3—Effect of freezing, freeze drying, atomization, heating inside the atomizer, and spray drying on cell survival after processing

	Batch no.		
	34	35	37
Cell paste (10^{10}CFU/g)	51 (7)[b] (n=2)[c]	51 (10) (n=4)	45 (7) (n=4)
Yield (g/L)[a]	4.71	5.43	4.93
Dry cell mass in paste (%)		21.8	22.5
Feed solution (10^9 CFU/mL)	13 (2) (n=8)	17 (1) (n=8)	17 (1) (n=8)
Treated cells (% survival)			
Atomized		107 (13) (n=4)	
Heated in atomizer		86 (7) (n=4)	
Spray dried (77°C)	63 (7) (n=4)	50 (8) (n=8)	74 (5) (n=8)
Frozen (−40°C)	116 (12) (n=12)	95 (9) (n=12)	102 (10) (n=12)
Freeze dried	93 (8) (n=12)	79 (8) (n=12)	91 (9) (n=12)

[a] Grams of cell paste per liter of fermentation culture.
[b] Mean (standard deviation).
[c] Plates counted for a single experiment, except for the frozen and freeze-dried cells for which the experiment was performed in triplicate and four plates were counted for each experiment.

Cell survival after dehydration

To study the effect of dehydration on cellular injury, a single feed solution with 3% cell loading was spray dried, frozen and freeze dried. Cell survival was measured before and after each treatment (Table 3). The experiment was performed in triplicate for each batch, using three batches of cell paste (no. 34, 35, and 37). The mean cell survival after spray drying at outlet-air temperature of 77°C was 62%. This was well within the range of previous results where the mean cell survival was 61% (Table 2). Thus, results for cell survival were highly reproducible for different experimental trials and batches of cells.

The mean cell survival after freezing and thawing was 104%. Survivals slightly greater than 100% could result from freezing and thawing action which might break-up chains of cells and increase the colonies counted on the plates. Nevertheless, the viability of *Lc. lactis* C2 was probably not affected by freezing and thawing. In corroboration, after freezing and thawing of this strain Speckman et al. (1973) measured 94% survival.

The mean cell survival after freeze drying was 88%. Freeze-dried samples contained ≤ 1% moisture, which may have decreased survival due to dehydration. The slight increase in cell counts due to freezing and thawing was eliminated by dividing the percent survival of frozen cells into the percent survival of freeze-dried cells. This resulted in a mean cell survival, after dehydration only, of 84%. Thus, cell dehydration caused a small but measurable decrease in survival for *Lc. lactis* C2. Speckman et al. (1973) measured 100% survival after freeze drying this strain.

Survival after spray drying was less than that after either freezing or freeze drying. This was apparently not due to shear in the atomizer. The combined effects of heating inside the atomizer (86%) and cell dehydration (84%) should produce 72% cell survival after spray drying. The lower (62%) cell survival after spray drying may have resulted from thermal inactivation of cells as droplets traversed the spray dryer, as described by Fu et al. (1994).

Cell viability after freezing and freeze drying depends on many factors such as the bacterial strain, cell loading, and processing conditions (Champagne et al., 1991). These factors probably also determine cell viability after spray drying, and the relative importance of dehydration and thermal inactivation. Some of these factors inevitably differ between different laboratories or times. Consequently, direct comparisons are difficult unless performed in one laboratory at one time, as was done in this study.

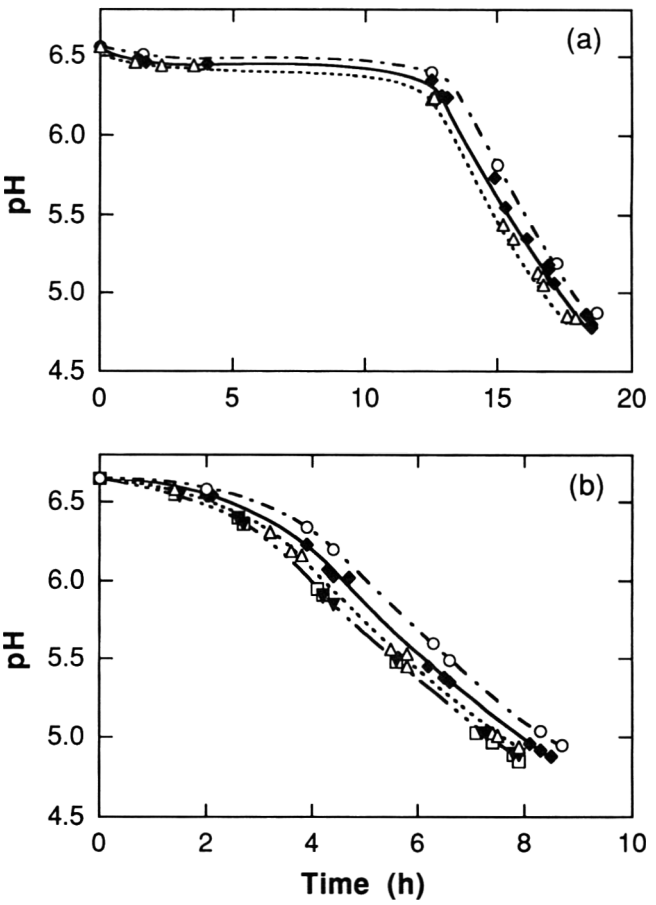


Fig. 4—Effect of processing on the drop in pH due to lactic acid production for cells in 9% nonfat dry milk solutions at 32°C: (--○--) spray dried cells; (——◆——) freeze dried cells; (---△---) frozen cells; (—□---) feed solution and (—▼---) cell paste. (a) batch no. 34, (b) batch no. 37.

Effect of cellular injury on lactic acid production

The drop in pH due to lactic acid production was related to time (Fig 4a) for the experiment of Table 3 (batch no. 34). The lag time before lactic acid production was 12 h for cells which were spray-dried at outlet-air temperature of 77°C, frozen or freeze-dried. The 12 hr lag time for spray-dried cells was identical to the previously determined value (Fig. 3(b)), obtained using a different batch. This indicated the results were reproducible. After the lag time, the rate of lactic acid production was equivalent for all samples.

We could hypothesize the time required to bring the viable cell counts in the milk dilution bottles up to a level required for significant lactic acid production caused the 12 to 16 hr lag times. In another hypothesis, long lag times could be due to repair of reversible cellular injuries prior to cell growth and lactic acid production. To test these hypotheses, lactic acid production was measured for duplicates of the experiment. In these experiments, lactic acid production was measured for the cell paste and feed solution, as well as the spray-dried, freeze-dried and frozen cells (Table 3, batch no. 35 and 37).

The drop in pH versus time due to lactic acid production [no. 37, Fig 4(b)] was compared. Lactic acid production for the other duplicate (no. 35) was very similar to these results (Fu, 1994). Unexpectedly, no lag times occurred before lactic acid production for any of the samples in either duplicate trial (no. 35 and 37). The initial viable cell counts in the milk dilution bottles were essentially identical (5×10^7 CFU/mL) in all experiments (Fig. 4(a) and 4(b)). If long lag times were simply the result of the time required to bring viable cell counts in the milk dilution bottles up to a level required for significant lactic acid production, then there should be long lag times in both Fig. 4(a) and

4(b). However, there were no lag times in Fig. 4(b). Consequently, the 12 to 16 hr lag times in experiments Fig. 4(a) were apparently due to repair of reversible cellular injuries prior to cell growth and lactic acid production.

Several possibilities were considered to better understand the absence of a lag time for no. 35 and 37 compared to the 12 to 16 hr lag times in previous experiments. No differences occurred between batches in viability of the cell paste, fermentation yield, solid content of the cell paste, or cell count of the feed solution (Table 3). All fermentation broths were made using the same lot numbers of components, and the same frozen culture. Potential variations in the 9% nonfat dry milk solutions, or the method used to measure lactic acid production, did not contribute to differences in lag time. Using the same milk solution at the same time, lactic acid production was measured for four-month-old spray-dried cells from batch no. 34, which again produced a 12 to 13 hr lag time, and for 5-day old spray-dried cells from batch no. 35, which again had no lag time.

Foster (1962) measured lactic acid production in milk for samples of cell paste and spray-dried powder of *S. lactis* C10. He reported that one lot of powder had a lag time 5 hr longer than that of the fresh culture. Two other lots of powder had lag times which were 0.5 to 1 hr longer than those of the fresh culture. These differences were apparent immediately after drying, and persisted throughout storage. Once growth began, both powder and fresh culture produced acid essentially at the same rate and to the same extent. Lactic acid production for the spray-dried cells and the fresh culture was an inherent characteristic of the particular culture. Those results were very similar to our spray drying results.

Because the production, freezing, freeze drying, spray drying, and analysis of cells were identical between experiments with short and long lag times, the reasons for the differences are not understood. It is unlikely all three methods simultaneously caused less cellular injury during both duplicate trials (no. 35 and 37). The characteristics of a cell paste for lactic acid production after freezing, freeze drying or spray drying were apparently established before processing, and were specific for each cell paste.

CONCLUSION

WHEN *LC. LACTIS* C2 CULTURES were spray dried at outlet-air temperature of 77°C using a cell loading in the feed solution of 20%, residual viability was 78%. The dried cultures lost 54% viability after storage for 91 days at 4°C. The mechanisms for cellular injury during spray drying were identified as: heating in the atomizer, cell dehydration, and thermal inactivation during drying. Shear in the atomizer did not cause cellular injury. When no lag times were observed for cell paste, feed solution, and frozen cells, then spray-dried starter cultures also had no lag times (batch no. 35 and 37). In other experiments, lag times of 12 to 16 hr were observed for cells which were either frozen, freeze-dried or spray-dried. Differences in lag times were apparently inherent for each specific cell paste. The lag time before lactic acid production from a single cell paste was essentially independent of treatments. Based on our results spray dried starter cultures could be prepared without notable reduction in viability or lactic acid production. Spray-dried cells sometimes produced lactic acid as rapidly as frozen cells. Consistent production of spray-dried cultures with short lag times depended on controlling characteristics of the cell paste.

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Biosensors for Analysis of Ethanol in Foods

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ABSTRACT

An amperometric ethanol sensor was constructed by co-immobilization of alcohol dehydrogenase (ADH) and bovine serum albumin (BSA) in carbon paste containing entrapped NAD^+. Conditions for optimum electrode response were determined by manipulation of experimental variables. Incorporation of both ADH and NAD^+ in carbon paste resulted in better relationships between the enzymatic reaction and the electrochemical process. The surface of such an electrode could be renewed by polishing and smoothing on a clean paper. The sensor showed rapid response time and high sensitivity.

Key Words: biosensors, carbon electrode, ethanol analysis, alcohol dehydrogenase (ADH)

INTRODUCTION

ETHANOL FROM SUGAR FERMENTATION may be added or formed in food as a part of its production. Ethanol formation may indicate an undesirable fermentation reaction affecting food quality. Thus the detection of ethanol may also be considered as an alert indicator. In such cases ethanol must be determined at the beginning of fermentation when the amount of ethanol is low. Classical methods, using for example distillation, are not applicable to such analyses, so sensitive methods using biosensors for ethanol assay may provide advantages.

The use of enzymatic reactions coupled with electrochemical detection has been an important part of the development of biosensors (Turner et al., 1987). This development was facilitated by the commercial availability of enzymes (Bartlett and Whitaker, 1987/88). Many strategies have been used to make enzyme electrodes: the enzyme may be covalently linked, adsorbed directly at the electrode surface or immobilized indirectly within an inert membrane fixed on the electrode surface. Progress made in the techniques of immobilization of enzymes have contributed to development of biosensors. However, the dehydrogenases, which require NAD^+ or NADH for reaction, have not often been used in biosensors (Gorton et al., 1993) due to the complexity of immobilizing both enzyme and cofactor. Indeed, contrary to oxidase for which the cofactor is bound to the enzyme, NAD^+ (or NADH) is not linked to the dehydrogenase (Gorton et al., 1991a). For the design of biosensors based on dehydrogenase, NAD^+/NADH must be kept close to the electrode surface to which the enzyme is attached. Addition of NAD^+/NADH to sample solutions greatly increases the cost of such sensors. In addition, the linkage of NAD^+/NADH directly at the surface of the electrode or via a membrane, strongly affects the biosensor sensitivity (Alberty et al., 1987; Sim, 1990). One of the best ways to overcome this is to incorporate NAD^+/NADH as well as the dehydrogenase into the conducting matrix of the electrode. In such case the matrix could be used as dehydrogenase sensors and could serve as a reservoir of cofactor (Zhao and Buck. 1991; Gorton et al., 1991b; Wang et al., 1992; Kulis et al., 1992).

Our objective was to demonstrate how the simultaneous entrapment of NAD^+ and ADH in carbon paste could be applied, if both components were uniformly dispersed within the matrix, to construct a biosensor with a renewable surface for measurement of ethanol.

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MATERIALS & METHODS

Chemicals and equipment

Alcohol dehydrogenase (EC.1.1.1.1. Boehringer, cat. No. 102 717), β-nicotinamide adenine dinucleotide reduced form, NADH (Fluka cat. No. 43 423), β-nicotinamide adenine dinucleotide oxidized form, NAD^+ (Sigma cat. No. N-7132), Bovine serum albumin, BSA (Fluka cat. No. 63 309), carbon powder (Carbon Lorraine cat No. 16858), ethanol standard solutions (Sigma cat. No. 332-11) and paraffin oil (Merck cat. No. 7161) were used as received.

Electrochemical measurements were made with EG & G Instruments (model 273) three-electrode potentiostat. A saturated calomel electrode (SCE) and stainless steel wire served as reference and counter electrodes. The ADH/NAD^+ modified carbon paste served as working electrode.

Electrode preparation

Ten mg of ADH and 10 mg of BSA were dissolved in 5 mL distilled water, then 50 mg of NAD^+ were added to the solution. For uniform dispersion of NAD^+ within the gel formed by the reaction of proteins and glutaraldehyde, the aldehyde was added slowly to the stirred protein-NAD^+ solution. Carbon powder was then added and the resulting mixture stirred for 5 min. This mixture was then dried in the refrigerator at 4°C. The resulting active carbon powder was further dried in a vacuum desiccator. Carbon paste was prepared by mixing, in a mortar, active carbon powder with liquid paraffin oil.

The carbon-paraffin oil paste was filled into a plastic tube with internal diameter 0.3 cm. Electrical contact was made by inserting a 0.27 cm diameter stainless steel rod in one end of the plastic tube. This rod was also used to push the paste out the other end of the plastic tube which was smoothed on paper to produce a flat electrode surface.

Procedure

The polished and smoothed electrode surface, renewed after each measurement, was immersed in the sample solution. Cyclic voltammetry experiments were run at 10 mV.s-1 as a potentials scan rate. Chronoamperometric experiments were run by applying a fixed potential of 0.5 V (vs SCE) at the working electrode and measuring the current vs. time (for a standard experiment t = 20 sec). After each experiment, the used active electrode surface was renewed by pushing the paste out of the plastic tube. The small quantity of evacuated paste was eliminated by polishing and smoothing the end of the electrode on a clean paper. All measurements were made at room temperature (20°C).

RESULTS & DISCUSSION

ONE WAY TO CONSTRUCT an ethanol biosensor is to modify the carbon paste electrode with NADH and ADH. The first step of the measurement would be the oxidation of NADH at the surface of the electrode in order to electrochemically generate NAD^+ (which reacts with ethanol in the presence of ADH to produce acetaldehyde and regenerate NADH). NADH could again be involved in the electrochemical oxidation process, leading to a pseudo EC' mechanism (chemical reaction, C, following the electrochemical one, E, and generating the starting electroactive species) (Bard and Faulkner, 1980). This type of biosensor could react in a similar way to that using glucose oxidase and electrochemical mediator.

The cyclic voltammograms (Fig. 1) of the carbon paste electrode modified with ADH and a very small amount of NADH

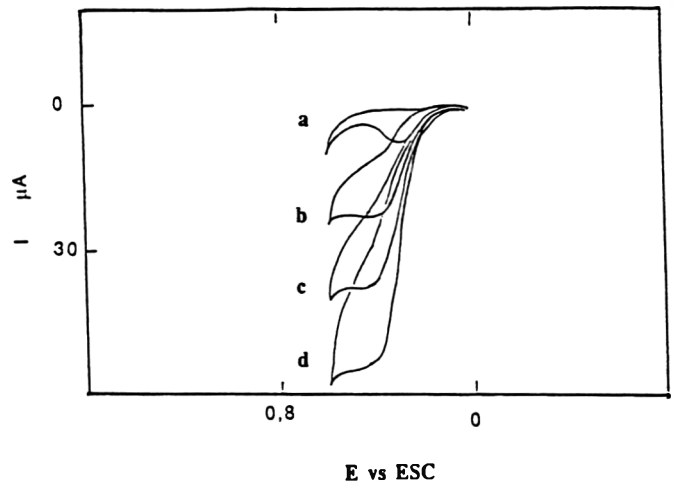


Fig. 1—Cyclic voltammograms of enzyme modified carbon paste electrode. In absence (a) and presence of increasing ethanol in solution (b) 0.8, (c) 4.8, and (e) 8.8% ethanol (v/v). The paste contained 0.5% NADH, 2.5 U ADH/mg (of paste) and 22% paraffin oil. Scan rate: 10 mV/sec, carbonate buffer 0.2M, pH = 10.0.

(0.5%) were compared in the absence (a) and in the presence of increasing amounts of ethanol in the solution (b, c, and d). The anodic current peak corresponding to oxidation of NADH increased when ethanol was added to the solution. This result was in agreement with the following mechanism:

$$NADH \rightarrow NAD^+ + H^+ + 2\,e^- \quad (1)$$

$$Ethanol + NAD^+ \rightleftarrows NADH + Acetaldehyde + H^+ \quad (2)$$

This result shows that the cofactor NAD^+ is still mobile and can rapidly diffuse from the paste electrode to the diffusion layer, where it reacts with ethanol and ADH.

Contrary to the EC' mechanism involved in the electrochemical oxidation of glucose with glucose oxidase via a mediator, the electrochemical step is slow. Furthermore the enzymatic step is an equilibrium shifted toward the NAD^+ formation in the presence of an excess of NADH. In addition the entrapment of NADH in the carbon paste would induce a high background current due to its electrochemical oxidation.

In order to overcome such disadvantages we added in the carbon paste, beside ADH, the cofactor in its oxidized form, NAD^+. The main improvements were a very notable decrease in background current and the possibility of adding the cofactor in relatively large amount (3%), in order to shift the chemical equilibrium toward NADH formation.

Five carbon pastes were prepared with different amounts of ADH, in the range 1–12 U of enzyme/g paste while the amount of NAD^+ was kept constant (3%). The sensitivity of the modified electrode was dependent on the amount of enzyme incorporated in the carbon paste (Fig. 2). The sensitivity seemed nearly constant for electrodes containing 5–9 U/mg paste. The increase of sensitivity for electrodes containing 1–5 U/mg could be explained by the increase of enzymatic activity of the electrode surface. The decrease of signal for the high amounts of enzyme was probably the result of accumulation of NADH in the diffusion layer (rate of formation of NADH via enzymatic reaction was faster than its oxidation at the surface of the electrode), leading to shift of equilibrium toward ethanol formation. At high enzyme concentration, the enzymatic oxidation of NADH by acetaldehyde would compete with its electrochemical oxidation. This is in agreement with the well known inhibitor effect of acetaldehyde on the rate of enzymatic oxidation of ethanol (Whitesides and Lee, 1985; Bourdillon et al., 1991). However, in an attempt to eliminate this inhibition, aldehyde dehydrogenase (AlDH) was added with ADH and NAD^+ to the carbon paste, but no effect was detected on electrode response. Again, no effect was found when AlDH was added into the ethanol solutions. The hypothesis of a blocking of the electrode surface by an excess of proteins could not be totally rejected to explain the decrease of the signal at high ADH rate (Wang and Hutchins, 1985). This effect could result either from the diminishing of the transducer area or by creation of a barrier to the diffusion of NAD^+ from the electrode to the solution (Gorton et al., 1993; Wilson and Sittampalam, 1991).

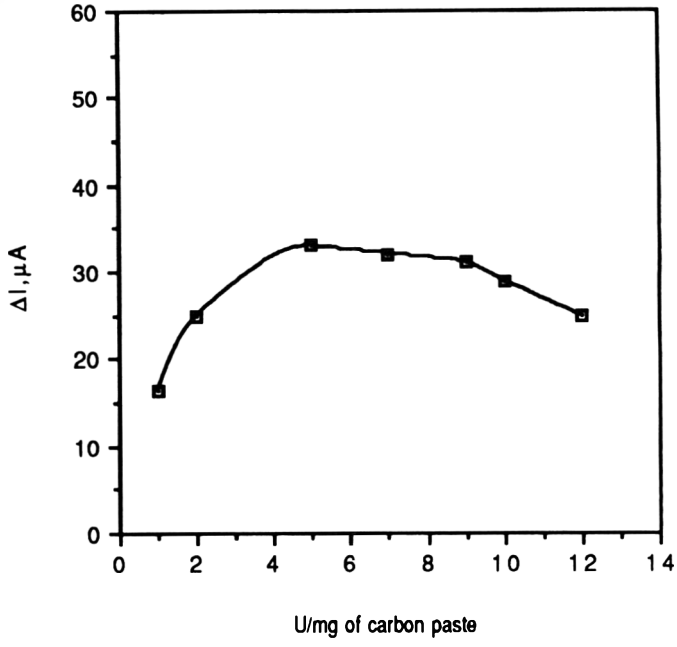


Fig. 2—Current as related to amount of ADH in 3% NAD^+ modified carbon paste. 0.2 M carbonate buffer, pH = 10.0. Ethanol concentration = 0.08% (v/v). Working potential : 0.5 V vs S.C.E. Current measured at t = 20 sec.

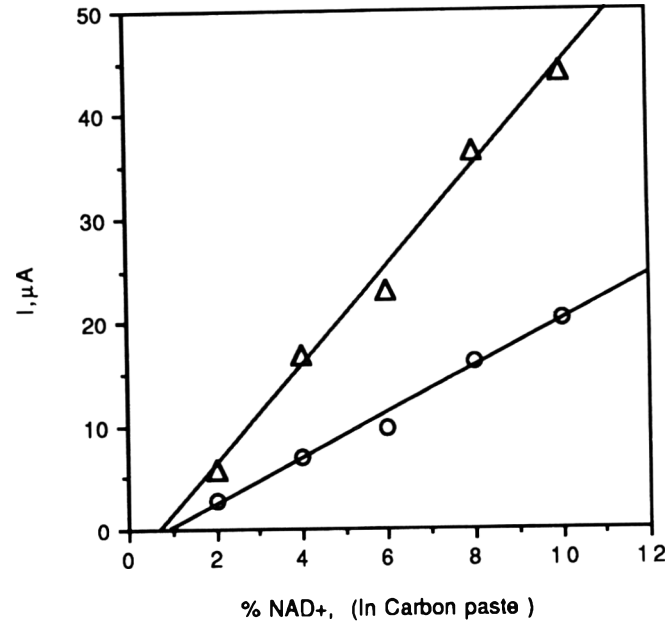


Fig. 3—Effect of percentage of NAD^+ on the ADH (2.5 U/mg) modified paste electrode (○) 0.02 (△) 0.06% ethanol (v/v) in 0.2 M carbonate buffer, pH = 10.0. The current is measured at 0.5 V vs S.C.E. t = 20 seconds.

Contrary to the effect of high protein rates or to observations in solution (Engstrom and Blaedel, 1980), increasing NAD^+ in the carbon paste caused an increase in electrode signal. The importance of the increase of measured current in relation to the increase of NAD^+ in the electrode was apparent (Fig. 3). The current was about 10 times higher when the NAD^+ rate increased

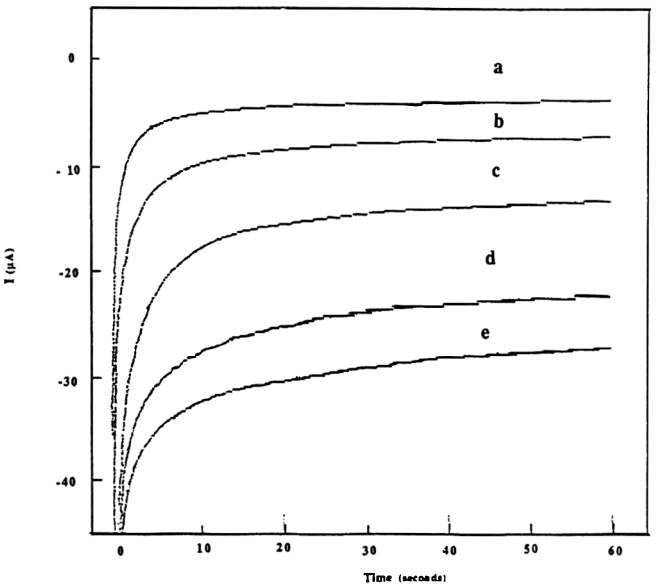


Fig. 4—Current as related to time. Paste containing : 6% NAD⁺ and ADH (5 U/mg). Carbonate buffer without (a), with : 0.005 (b), 0.01 (c), 0.03 (d), and 0.05% (v/v) ethanol (e). Working potential : 0.5 V vs S.C.E.

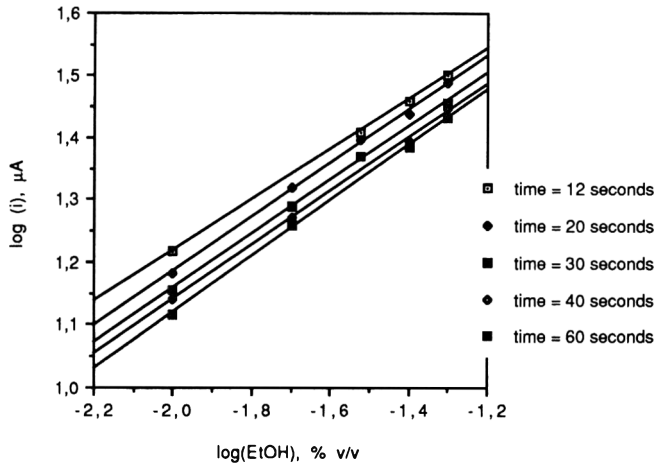


Fig. 5—Current as related to ethanol concentration at different measuring times. Paste modified by 6% NAD⁺ and 5 U ADH/mg. Working potential : 0.5 V vs S.C.E.

Table 1—Check for carbon paste homogeneity (Carbon paste contains 8.5% NAD⁺ and 8 U ADU/mg)

Ethanol conc (mM)	Mean[a] current intensity (µA)-	Maximum current intensity (µA)	Minimum current intensity (µA)	S.D	R.S.D.[b] (%)
1.72	18.1	18.2	17.8	1.3	0.7
8.24	40.8	41.4	39.8	0.6	1.4
11.16	47.8	48.9	46.9	0.8	1.7

[a] Mean for six successive measurements of each sample.
[b] Relat ve standard deviation.

from 1.5% to 8%. This was because of the very high concentration of NAD⁺ near the electrode surface which drove the enzymatic reaction towards acetaldehyde and NADH formation. The electrode worked as a NAD⁺ reservoir and continuously supplied ADH with its cofactor. Here, electrochemical regeneration of NAD⁺ was no longer the limiting step for the enzymatic oxidation of ethanol.

Another advantage in using the electrode as NAD⁺ reservoir is that the electrode response would be independent of incubation time (the moment the electrode surface is in contact with a

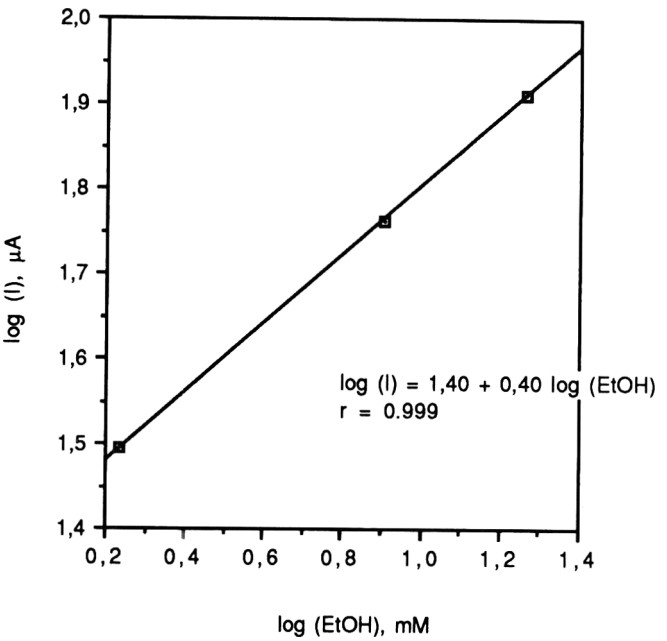


Fig. 6—Calibration curve. Current measured at 0.5 V vs S.C.E. t = 20 seconds. Carbon paste containing 8 U ADH/mg and 8.5% NAD⁺. The internal diameter of the working electrode is 0.3 cm. Ethanol solutions in carbonate buffer; pH = 10.0.

solution containing ethanol to beginning of measurement). This was in agreement with a very fast establishment of equilibrium between ethanol and NAD⁺ on one hand and acetaldehyde and NADH on the other hand. Electrode signal was proportional to ethanol concentration via its relation to NADH oxidation.

Considering Eq (1) and (2), we could readily recognize the very important effect of pH on the process. pH affected the stability of both NAD⁺ and NADH, the activity of ADH and the equilibrium which involved H⁺. On the other hand, it has been shown that the electrochemical oxidation of NADH was not pH dependent (Samec and al., 1982). We studied the effect of pH on electrode response at different ethanol concentrations. We found that, whatever the ethanol concentration, sensitivity of the electrode increased when pH increased. This was in agreement with the use of ADH, in high pH solutions for oxidation of ethanol. But contrary to other reports (Danielson and Wangsa, 1991), we noted no decrease in electrode sensitivity when pH was higher than 9. This was probably due to the fact that, at the electrode surface, the pH was lower than in the bulk of the solution which would result in a higher stability for ADH and for its cofactors. The very short measuring time ($t < 20$ seconds), also contributes to the stability of enzyme activity during analysis.

The current vs electrode potential was also studied in ethanol solutions of different concentrations. For potentials of 0.5 V to nearly 1.2 V vs S.C.E. the measured current was constant. For further experiments, the potential of the electrode was fixed (chronoamperometric experiments) at 0.5 V to avoid interfering reactions.

The response of the electrode (potential : 0.5 V vs SCE) as a function of time for different ethanol concentrations was followed (Fig. 4). The stabilization of the current occurred very rapidly and measurement could be achieved in a few seconds. A very short measuring time (12 sec) only affects the intercept of the calibration curve (Fig. 5) slopes were almost identical if measuring time changed from 12–60 sec.

Because problems in paste homogeneity could be encountered when mixing components in electrode fabrication, we investigated the reproducibility of our electrode in two different ways. We measured the same ethanol solution and analyzed statistically the resulting current values, with each value obtained with a new clean and wiped electrode surface (Table 1). For this

Table 2—Repeatability of ethanol measurements (Electrode modified with 8.5% NAD^+ and 8 U ADH/mg)

Reference[a] conc (mM)	Measured[b] conc (mM)	R.S.D. (%)	% Error
4.98	4.88	3.30	-2.13
12.70	12.91	0.55	-1.60

[a] Solutions obtained by dilution of standard sample.
[b] Mean of three successive measurements for each standard sample.

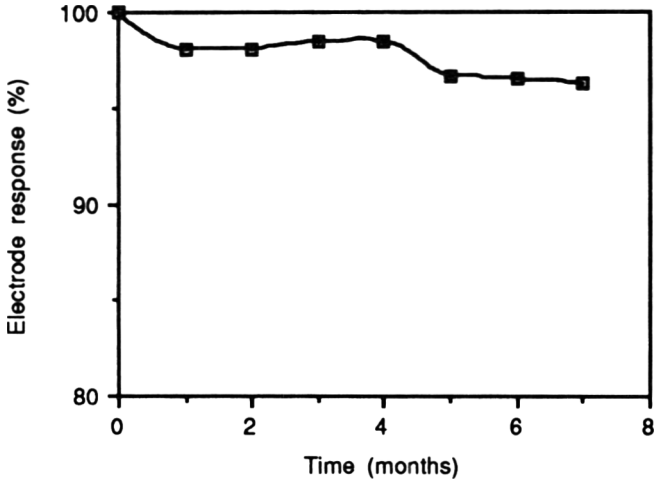


Fig. 7—Shelf lifetime for ethanol electrode. Carbon paste modified with 6% NAD^+ and 5 U ADH/mg.

study exact ethanol concentrations were not needed, and our results showed a very good reproducibility of the electrode.

We also investigated the reproducibility of the electrode by assessing the values obtained while we were investigating the accuracy of this electrode. The reproducibility was not only related to currents but to concentrations. That it is why we used 3 ethanol standard solutions : 1.7, 8.1 and 18.4 mM to plot the calibration curve (Fig. 6), which was used to read the concentrations of 2 ethanol solutions at 4.98 and 12.70 mM. For each measurement the electrode surface was renewed and wiped before use (Table 2). Results showed very good reproducibility and accuracy of our enzyme modified electrode.

The loss in activity of the ADH/NAD^+ modified paste, after 7 months (Fig. 7) was less than 5% when the electrode was stored, without any other specific care, at +4°C.

CONCLUSION

INCORPORATION OF BOTH NAD^+ and ADH in the carbon paste could be used for preparation of alcohol biosensors. The renewable electrode surface and fast response make this type biosensor simple to use and gave reproducible and accurate results. This sensor was stable for several months and remained readily available. The carbon paste electrode could also be miniaturized and may be used as a portable and easy-to-handle biosensor. Other dehydrogenase-type biosensors are being tested.

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Permeability and Diffusivity of d-Limonene Vapor in Polymeric Sealant Films

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ABSTRACT

The permeability (P) and Diffusivity (D) of d-limonene vapor in nine polymeric sealant films were measured and compared at a vapor concentration of 0.719 mg/L over the range 25–45°C. The sorption of d-limonene (q_s) was determined by a temperature programmed desorption (TPD) method. Equations to predict P and D for the different films were derived as a function of q_s or temperature (T_{mp}) of differential scanning calorimeter measured melting points (DSCmp) of the films. Results indicated a tendency of test films with smaller values of q_s and higher T_{mp} to have higher barriers against loss of d-limonene due to absorption.

Key Words: d-limonene, permeability, diffusivity, polymeric films

INTRODUCTION

POLYETHYLENE-DERIVED FILMS have been widely used on the inner surface of polymer containers. An important problem is the sorption of flavor compounds from food into such films (Sadler and Braddock, 1990; 1991). Imai et al. (1990) reported that the decrease in d-limonene (a major volatile component in orange juice) was 15% of the total amount for an ethylene vinyl alcohol copolymer of high ethylene content (EVOH) and 41% for a low density polyethylene (LDPE) during 5 days storage. Kwapong and Hotchkiss (1987) reported that the extent of sorption depended on type of polymer, concentration and chemical structure of the compound, storage temperature and time, and other components in the aqueous phase. These factors complicate the prediction of permeability, diffusivity and solubility of d-limonene in polymeric sealant films.

Mohney and Hernandez (1988) conducted well designed experiments with d-limonene vapor, to evaluate quantitatively the *permeance* (P) in a high density polyethylene (HDPE)/sealant laminate, as a function of vapor concentration of d-limonene (*Cv: mg/L less than 5.5mg/L*). The equation obtained was

$$P = 4.9 \times 10^{-3} \exp(0.7 \cdot C_v) \text{ for HDPE (83\%)} \quad (1)$$

P: g·structure/m²·day·ppm

The equation must be derived for all test films. Generally the measurements of P and D need long periods of time to evaluate transmission rate profile curves of d-limonene though permeation depends on concentration. It would be useful if P and D could be approximated by some film physicochemical parameters. Our major objectives were to derive equations for evaluating permeabilities of various polyethylene derived films and other types of films as related to solubility of d-limonene and the differential scanning calorimeter measured melting point (DSCmp) of the films.

MATERIALS & METHODS

Materials

D-limonene was supplied from Wako Chemical Company (purity 90%). Three polyethylene derived sealant films (PE-group) were used: low density polyethylene (LDPE), linear low density polyethylene (LLDPE), and high density polyethylene (HDPE), and two ethylene vinyl acetate copolymers (EVA-group, the amounts of vinyl acetate were 5wt% (EVA5) and 7wt% (EVA7)).

Three composite films (EAA-group) of the random copolymer of low density polyethylene acrylic acid (EAA) mixing with polyethylene telephthalate (PET) were used: EAA:PET = 1:2, 2:1 and 1:0, designated EAA33, EAA67, and EAA100. A reference material characteristic of high barrier materials, polyacrylonitrile copolymer film (PAN), was used. All test films were provided by the Tama-poly company. The chemical structures and characteristics of the films were tabulated (Table 1) with density and degree of crystallinity of the films.

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Permeability

The permeability measurement cell was the same as that reported by Hernandez et al. (1986). A schematic diagram (Fig. 1) represents the isostatic test apparatus. It consisted of two stainless steel chambers, with upper and lower cell chambers each with a volume of 3.6 cm³. The cell was assembled by mounting the film to be tested between two stainless steel plates. The permeation area of the film exposed to d-limonene vapor was 18 cm². Hermetic isolation of the chambers was achieved by compression of overlapping silicon rubber "O" rings on the film sample.

The permeation cell was immersed into a water bath with temperature controlled ±0.1°C. D-limonene vapor was prepared by bubbling nitrogen gas through liquid d-limonene at 30°C. To prepare the lower concentration (0.719mg/L), the permeant vapor stream was diluted with another stream of nitrogen. The d-limonene vapor prepared thus flowed through the upper cell chamber and pure nitrogen gas flowed through the lower cell chamber removing permeate vapor at a constant rate (75 mL/min).

The concentration of upper and lower streams was monitored at the inlet and outlet sampling holes of the chambers by a gas chromatograph equipped with dual flame ionization detector (FID). A stainless steel column (1m, 3.18mm o.d.) was packed with Thermon-1500. The carrier gas was N_2 and flow rate was 30 mL/min. The injection port was 180°C and detector 100°C. Retention time was 35 sec. for d-limonene. The data are average values of two runs, and reproducibility in P was ± 5% and lag time was ± 20%.

Sorption

The sorption amount of d-limonene was measured by the temperature programmed desorption (TPD) method. 50 cm² film samples were stored in 17.8 mL Pyrex glass tubes and exposed to a d-limonene vapor flow of 30 mL/min for 24 hr at the test temperature. At this condition we reconfirmed that the absorption was in equilibrium for PE-, EVA-, and EAA- groups, though the equilibrium absorption for PAN required more than 1 wk.

The tube was flushed for 10 min by a nitrogen flow of 75 mL/min and the temperature was raised at 3°C/min to 180°C. The concentration of desorbed d-limonene was analyzed by gas chromatography and the total amount desorbed was evaluated by the graphical integration of the TPD curve.

Melting Points of Films

The melting point (T_{mp}) of sealant films was measured by using the endothermic peak of differential scanning calorimetric (DSC-8001, Rigaku Denki Co.) profile curve. The rate of temperature rise (α) was constant 10°C/min for all tested films, and T_{mp} did not vary significantly for other values of α.

Table 1—Characteristics of polymeric sealant films

Mark	Structure	Thickness (μm)	Density (g/cm^3)	DSCmp (°C)	Crystallinity x(%)[a]
LDPE	$(CH_2—CH_2)_n$	60	0.923	112	50.4
LLDPE			0.930	126	55.3
HDPE		55	0.952	131	70.0
EVA5	$(CH_2—CH_2)_m—(CH_2—\underset{OAc}{CH})_n$	70	0.928	107	—
EVA7				100	
PAN	$(CH_2—CH)_l—(CH_2—CH)_{n'}$ (CN, Me) $(CH_2—CH=CH—CH_2)_n$	30	1.150	—	—

[a] Evaluated by x (%) = $[(d-d_a)/(d_c-d_a)] \cdot [d_c/d] \cdot 100$ where d_a=0.856(g/cm^3), d_c=1.0(g/cm^3), and d is experimental value (g/cm^3).

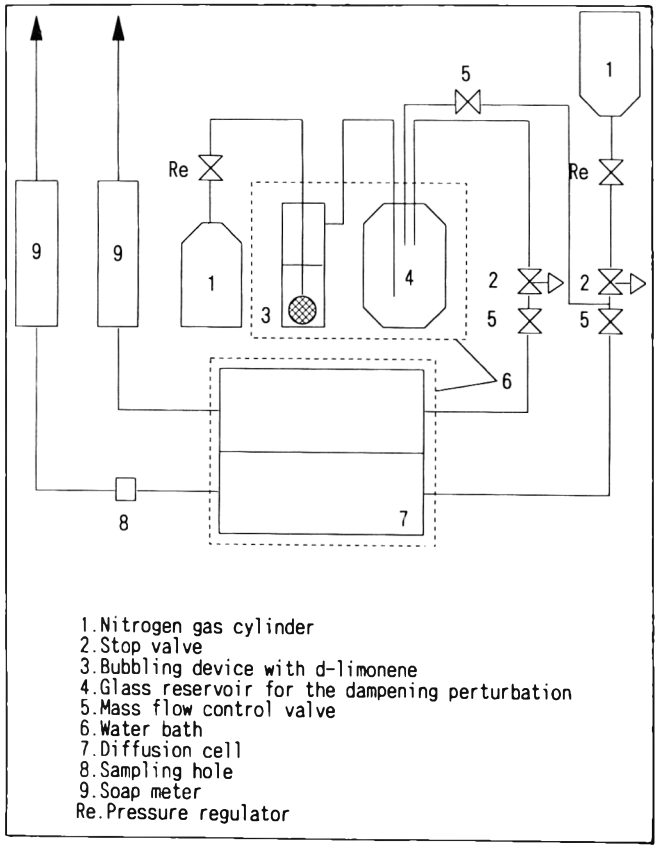


Fig. 1—Experimental apparatus.

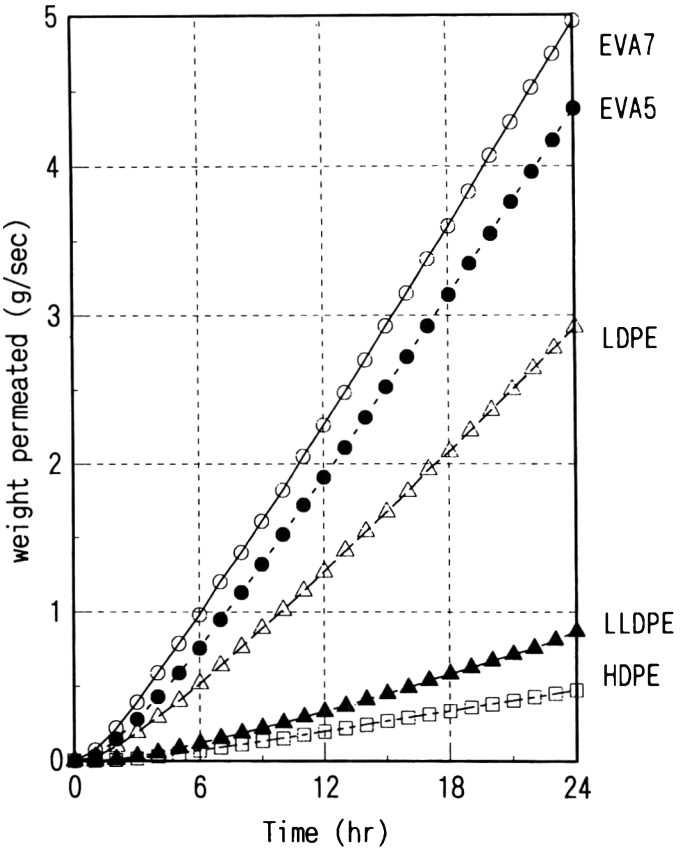


Fig. 2—Transmission rate profile curves for d-limonene through the test films at 25°C.

RESULTS & DISCUSSION

Permeability of d-limonene

The apparent permeability coefficient (P) was calculated from

$$P = \frac{Q \cdot L}{A \cdot (p_1 - p_2)} \quad (2)$$

where Q is the rate of permeation, L is the thickness of the film, A is the effective permeation area and p_1 and p_2 are partial pressures of d-limonene in the upper cell chamber (high concentration) and in the lower cell chamber where the permeate vapor was removed at a constant rate. The apparent diffusion coefficient D was calculated from the lag time method proposed by Barrer (1939) as follows:

$$D = \frac{L^2}{6 \cdot \theta} \quad (3)$$

where θ is the lag time.

The apparent solubility coefficient S was calculated from

$$S = \frac{P}{D} \quad (4)$$

As demonstrated by Mohney and Hernandez (1988), the permeability, diffusion and solubility coefficients of d-limonene depend on the test concentration of d-limonene. In our study, the concentration of d-limonene was held constant at 0.719 mg/L and the values of the three coefficients were compared between membranes.

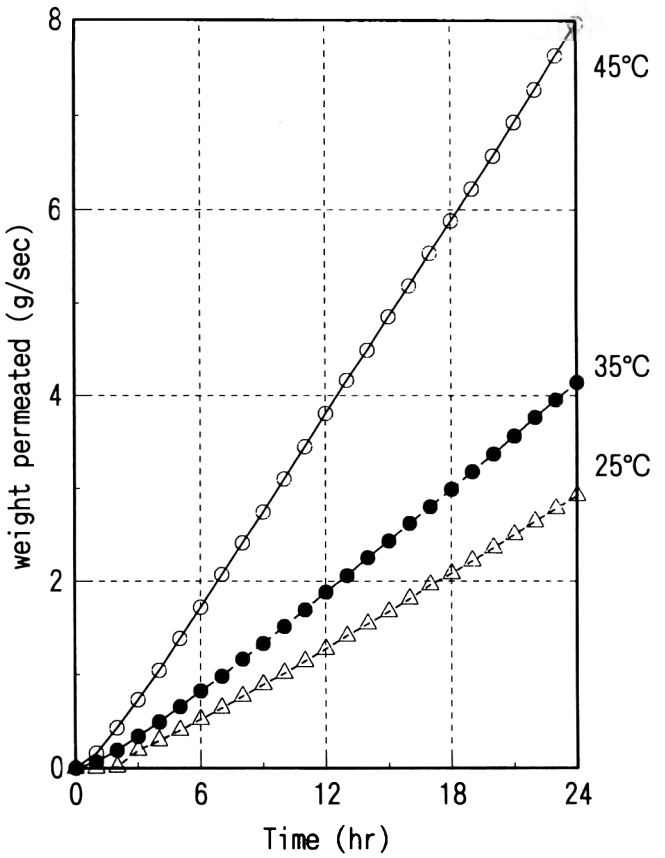


Fig. 3—Transmission rate profile curves for d-limonene through LDPE as related to temperature.

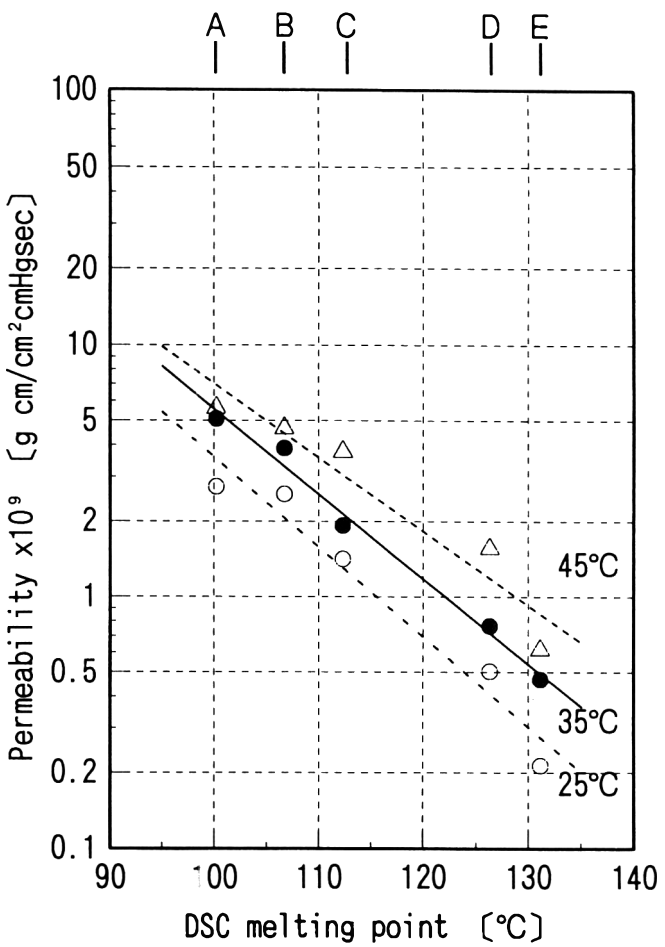


Fig. 4—Permeability as related to DSC melting point for test films. A=EVA7, B=EVA5, C=LDPE, D=LLDPE, E=HDPE.

Typical transmission rate profile curves for d-limonene through various PE-group and LDPE films were compared as a function of temperature (Fig. 2, 3). The two parameters measured for all films were summarized (Table 2) including the two parameters evaluated by using the equation of Mohney and Hernandez (1988) for comparison. Equation (1), for evaluating the P of HDPE as related to vapor concentration of d-limonene, was derived for concentrations < 5.5 mg/L. Using the equation, P at 0.719 mg/L of d-limonene vapor could be calculated at 7.11×10^{-11} g·cm/cm²·cmHg·sec which was one-third of our experimental value of 2.13×10^{-10} g·cm/cm²·cmHg·sec at 25°C. This may be attributed to differences of content of HDPE (83%) and measuring procedures and conditions (23°C). For PAN, the absorbed amount was 8.63×10^{-6} g-d-limonene/g-membrane, much less than other films indicating the high barrier property of the PAN film.

The apparent melting point of films could be evaluated by the differential scanning calorimeter (DSC melting point = DSCmp Table 1). The permeability coefficient (P) varied exponentially with DSCmp dependency for polyolefin and EVA containing films (Fig. 4). A least squares fit gave the following equation

$$P = 2.3 \times 10^{-3} \exp(-8.2 \times 10^{-2} T_{mp}) \quad (5)$$

P:g cm/cm²·cmHg·sec

where T_{mp} is the temperature of DSCmp (°C). Although EVA5 and EVA7 are polyethylene copolymers containing 5 and 7 wt% of vinyl acetate respectively, they fit the same straight line within -0.945 of the correlation coefficient. The content of vinyl acetate in polyethylene structure thus increased the diffusion coefficient of d-limonene. From a macroscopic standpoint, assuming a simple two-phase structure of crystalline and amorphous regions in the PE-group films as demonstrated by Michaels and Bilxer (1961), the higher crystalline fraction of the polymer would give the higher T_{mp}. Based on this consideration, the crystalline portion of the test films may be ordered HDPE > LLDPE > LDPE > EVA5 > EVA7, the inverse order of the P values for the films. For PE-group films, the degree of crystallinity could be evaluated by using the measured density (Table 1) which was consistent with the above order.

Table 2—Permeability and diffusivity coefficients of d-limonene in films at vapor concentration C.719 mg/L

Film	Temp (°C)	Permeability[a]	Diffusivity[b]
EVA7	25	2.74	0.116
	35	5.08	0.136
	45	5.61	0.189
EVA5	25	2.56	0.081
	35	3.88	0.136
	45	4.67	0.194
LDPE	25	1.41	0.051
	35	1.92	0.067
	45	3.76	0.139
LLDPE	25	0.504	0.046
	35	0.765	0.050
	45	1.56	0.084
HDPE	23	0.0711[c]	0.0116[d]
	25	0.213	0.037
	35	0.467	0.053
	45	0.614	0.076
EAA33	45	0.0316	—
EAA67	45	0.527	—
EAA100	45	1.01	—

[a] P:×10⁻⁹ (g cm/cm²cmHgsec)
[b] D:×10⁻⁸ (cm²/sec)
[c,d] Values evaluated by Mohney et al's equation at 23°C.

Solubility of d-limonene by temperature programmed desorption (TPD) method

Some desorption curves of d-limonene for the films were compared (Fig. 5). The graphical integration of the curves gave

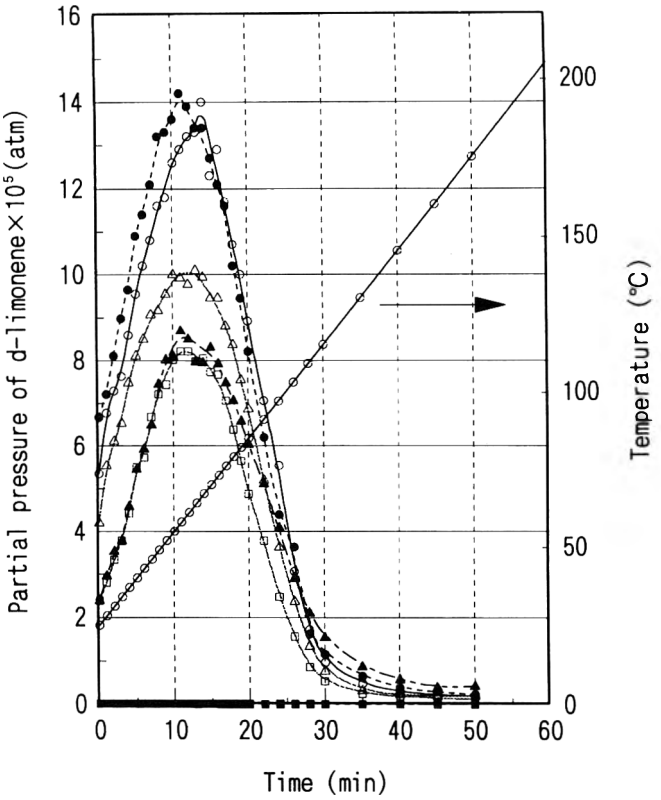


Fig. 5—TPD spectra of d-limonene from test films heated at 3°C/min after exposing films to d-limonene vapor of 0.719 mg/L at 25°C for 24 hr. ○—○ EVA7; ●---● EVA5; △·····△ LDPE; ▲----▲ LLDPE; □·····□ HDPE; ■—■ PAN.

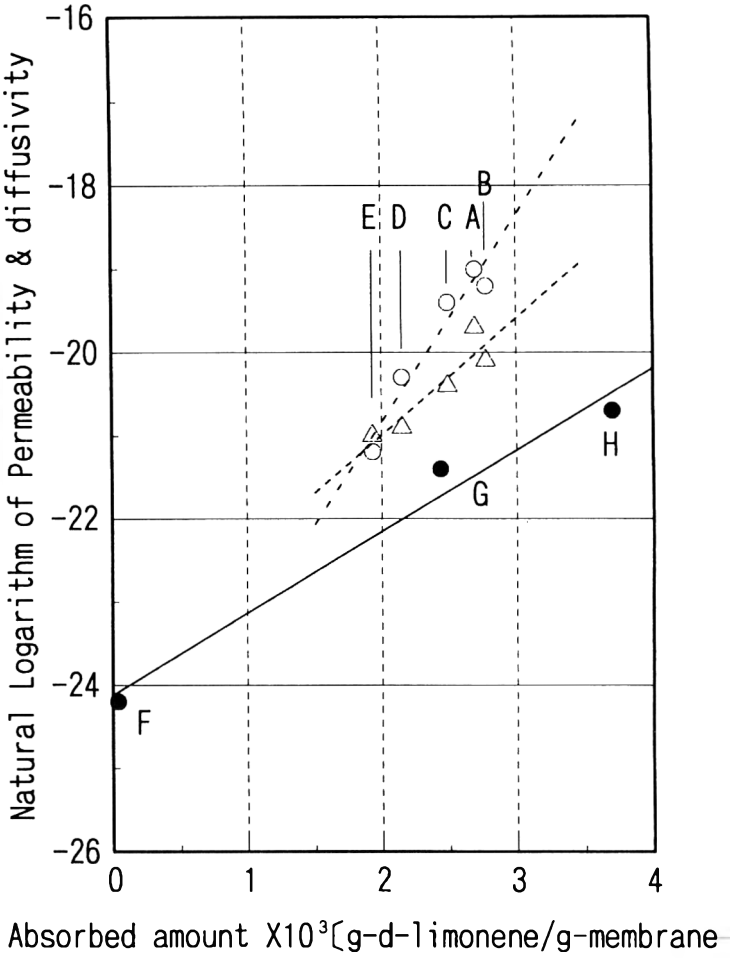


Fig. 6—Permeability and diffusivity of films as related to absorbed d-limonene. ○,△:A=EVA7, B=EVA5, C=LDPE, D=LLDPE, E=HDPE; ●:F=EAA33, G=EAA67, H=EAA100.

the amount of absorbed d-limonene at the given conditions (Table 3). The permeability coefficient and diffusion coefficient for PE-group showed an exponential concentration dependency of absorbed amounts (Fig. 6). From a least squares fit, the following expressions were derived at 25°C.

$$P = 6.2 \times 10^{-12} \exp(2.5 \times 10^{3} \cdot q_s) \quad (6)$$

$$D = 3.3 \times 10^{-11} \exp(9.8 \times 10^{2} \cdot q_s) \quad (7)$$

P:g cm/cm² cmHg sec

D:cm²/sec

where q_s (g-d-limonene/g-structure) is the amount of d-limonene absorbed at constant vapor concentration 0.719 mg/L for each film. The films were exposed on both sides to vapor, whereas they were exposed on one side only at the conditions of permeability measurement. Therefore q_s is not the same as for permeability measurement.

A similar procedure was also applied to composite films of EAA and PET (Fig. 6). The equation was derived as follows at 45°C:

$$P = 4.6 \times 10^{-11} \exp(1.4 \times 10^{3} \cdot q_s) \quad (8)$$

The apparent permeability coefficient decreased with increasing content of PET in the random copolymer of low density polyethylene acrylic acid, indicating the PET film was a more effective barrier against loss due to d-limonene absorption. For d-limonene absorption, the value of P. EAA33 was about 1/100 and 1/30 that of EAA100 not 1/3. It is difficult to explain this discrepancy. A possible explanation could be the strong mixing effect of the two polymer structures lowering the values of q_s and P. Compared to the PE-group it gave a much lower permeability coefficient though the absorbed amount in EAA100 was higher than EVA7. From this result, permeability was not dependent only on the absorbed d-limonene. The permeability coefficient could be influenced by the type of polymer structure (Kwapong and Hotchkiss, 1987).

Table 3—Absorbed d-limonene for various films at 25°C

Film	Absorbed amount[a]
EVA7	2.69
EVA5	2.77
LDPE	2.49
LLDPE	2.15
HDPE	1.93
PAN	0.00863
EAA33	0.0373
EAA67	2.43
EAA100	3.70

[a] Absorbed amount ×10³ [g-d-limonene/g-membrane]

Mohney and Hernandez calculated the solubility constant (K) for HDPE and Glassine structures derived from Henry's Law equation.

$$C_S = KC_V \quad (9)$$

where C_S is the solubility of d-limonene in the polymer (g-d-limonene/g-structure), C_V is the vapor concentration < 5.5 mg/L and K is a constant. They evaluated the value of K to be 7.3×10^{-3} g-d-limonene/g-structure ppm-d-limonene at 23°C for HDPE (83%). In our case, K was evaluated to be 2.7×10^{-3} at 25°C and a concentration of d-limonene of 0.719 mg/L. This discrepancy may be caused by the difference in films and the procedure of solubility measurement. The values of K for the PE- and EVA-groups films were also calculated and ordered as follows:

$$EVA5(3.85\times10^{-3})>EVA7(3.74\times10^{-3})>LDPE(3.46\times10^{-3})>LLDPE(2.99\times10^{-3})>HDPE(2.69\times10^{-3})$$

For the EAA-group films, we found as follows:

$$EAA100(5.15\times10^{-3})>EAA67(3.38\times10^{-3})>EAA33(5.19\times10^{-5})$$

These were the inverse orders of the barrier quality of sealant films vs loss due to d-limonene absorption and melting temperature of films. An exception was the EVA-group which showed higher K (or T_{mp}) values indicated lower permeability.

CONCLUSIONS

FOR BARRIER QUALITY for the loss of d-limonene the apparent permeability coefficient indicated the order of EVA > PE > EAA > PAN. In PE- and EAA-groups, the higher solubility of d-limonene gave higher permeability coefficients such as LDPE > LLDPE > HDPE for PE-group and EAA100 > EAA67 > EAA33 for EAA-group. PAN and PET were effective barrier films against loss of d-limonene due to absorption. The general equations to predict P and D values were separately derived as functions of T_{mp} for PE- and EAA-groups. Equations derived in this study can be used to evaluate barrier property of films from measured DSC melting point data.

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Policies for the Preparation and Review of Papers Reporting Sensory Evaluation Data

☐ THE SENSORY EVALUATION DIVISION of the Institute of Food Technologists has prepared the following recommended policies and guidelines for use by authors in preparing manuscripts which include sensory information, as well as for use by reviewers when evaluating the suitability of such manuscripts for publication.

Any papers containing sensory information that are submitted for publication in the *Journal of Food Science* should include the information given here. This listing is a guide. It is not intended to be exhaustive; however, the overriding concern is that the results should be applicable to foods (and beverages), directly or indirectly. If not, then the author(s) must relate their results to food. Inasmuch as there are other publications that accept manuscripts about sensory evaluation, including experimental psychology and physiology, the applicability to food will be rigorously applied. Acceptable papers will fall into one of two categories:

1. Papers that report the results of a sensory analysis of foods, beverages, or ingredients used in the preparation of foods and beverages are appropriate, as are those dealing with food concepts or model systems.

2. Papers that report methodology, statistical analyses and/or studies of the psychological aspects of the testing process that are applicable to the evaluation of foods, etc. as noted in item 1, will be considered. In the discussion of results, authors must make clear this link with the evaluation of food.

The following guidelines are similar to those listed in an earlier version (IFT, 1981) and, like it, this one is intended to help authors who are reporting results from a sensory test or are contemplating using sensory evaluation in their investigations. This is not a complete listing of all possible methods or procedures, and authors are encouraged to provide complete information about their research. As the IFT (1994) "Style Guide for Research Papers" specifies, one should "provide sufficient detail of the method and equipment to allow the work to be repeated." Berry and Noyes (1993) give additional general guidance for preparing a scientific paper.

When using procedures described in this guide or in other pertinent IFT publications, authors should cite the appropriate references and also provide sufficient detail to enable the reader to understand the procedures without continual reference to the literature. These guidelines are not intended to provide advice about sensory practice. A limited number of references are cited here as an introduction to the literature and are intended as an aid to authors and reviewers in obtaining more information about sensory evaluation methods and procedures.

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These guidelines were prepared by a committee of the IFT Sensory Evaluation Division consisting of Barbara P. Klein, Herbert Stone, and Harry Lawless and approved by the Division's Executive Committee.

Guidelines for the Preparation and Review of Papers Reporting Sensory Evaluation Data

by the IFT Sensory Evaluation Division

I. Abstract (if necessary)[a]
The abstract, which should not exceed 110 words, should convey the objective of the reported research, a brief description of the experimental procedures, results, and conclusions.

II. Introduction (approximately 500 words)
- A. Short review of pertinent research (with references)
- B. Definition of the problem
- C. Approach to solution of the problem
- D. Specific objectives for the sensory portion[b] of the study, in addition to the overall objective of the study

III. Materials & Methods
- A. Experimental design
  1. Explain the design (layout of the experiment) in terms of the objectives
  2. Reference the source of the design (statistics book, e.g., Cochran and Cox, 1957), including, as pertinent:
     a. Blocking (balanced, randomized, complete)
     b. Randomization
     c. Factorial designs
     d. Latin Square
     e. Other (describe)
  3. Measurements made (sensory[b], chemical and instrumental)
  4. Sample variables and levels
  5. Number of replications by subject
  6. Data manipulation (transformation, normalization)
- B. Sensory methods
  1. Analytical testing
     a. Difference testing
        (1) Forced-choice tests (e.g., m-AFC, triangle, duo-trio, paired comparison)
        (2) Multiple sample difference tests
        (3) Threshold
        (4) Ranking
        (5) Overall degree of difference test
        (6) Other (describe)
     b. Descriptive sensory profiling
        (1) Flavor Profile®
        (2) Texture Profile®
        (3) QDA Method®
        (4) Spectrum Analysis®
        (5) Time-Intensity
        (6) Any method in which attributes are rated (describe)
  2. Consumer and acceptance tests (Affective)
     a. Preference tests
        (1) Paired preference
        (2) Rank preference
        (3) Multiple paired or ranked
     b. Acceptance tests
        (1) Acceptability
        (2) Hedonic rating
        (3) Just-about-right scaling
        (4) Attribute ratings (diagnostics)
           (a) Hedonic
           (b) Intensity
        (5) Other (describe)
- C. Panel
  1. Number of subjects
  2. Selection and panel management
     a. Criteria
        (1) Employee/nonemployee (describe)
        (2) Demographics (age, sex, product usage for acceptance panels)
        (3) Ability to discriminate and whether reliability estimate was obtained
        (4) Criteria used to eliminate panelists or data (describe)
     b. Informed consent statement (if appropriate)
     c. Panelist incentives
     d. Other (describe)
  3. Training
     a. None
     b. Trained
        (1) Method
        (2) Duration
        (3) Use of references[c]
- D. Test conditions (include relevant units for conditions)
  1. Test area
     a. Location (booth, store front, shopping mall, home, etc.)
     b. Environment control (e.g., humidity as % RH, temperature)
     c. Lighting description (e.g., intensity, spectral distribution)
  2. Sample description
     a. Type of material
     b. Source of material
     c. Processing conditions
     d. Storage conditions
  3. Sample presentation
     a. Preparation methods
     b. Sample handling
        (1) Quantity (size, weight, volume)
        (2) Container
        (3) Utensils
        (4) Temperature of sample at serving
     c. Coding/labeling
     d. Order of presentation
     e. Number of samples evaluated per session
     f. Instructions to panelists (not all are applicable to all tests)
        (1) Ballot/scorecard (scale type, e.g., category, unstructured line, time-intensity)
        (2) Carrier
        (3) Expectorating, swallowing, rinsing
        (4) Time intervals between samples
        (5) Other (describe)
     g. Data collection method (computer system and software if used)
- E. Statistical techniques (refer to III.A. Experimental design)
  1. Specific type of analysis (Include statistical model and software used)
  2. Level of significance for rejection of hypothesis
  3. Transformation/conversion of data (describe)
  4. Post-hoc tests
  5. Correlations with other types of information (e.g., physical/chemical analyses, formulation and/or processing variables)
  6. Other (describe)

IV. Results & Discussion[c]

V. References[c]

[a]Abstract required for *Journal of Food Science*, not required for *Food Technology*

[b]Only the sensory portion of the study will be referred to in the remainder of these guidelines.

[c]Refer to IFT (1994) Style Guide for Research Papers

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