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19 ก.ค. 2527

Letters

ENZYME CROSS-LINKING OF FOOD PROTEINS

This correspondence is in reference to an article entitled "Transglutaminase catalyzed crosslinking of myosin to soya protein, casein, and gluten" by L. Kurth and P.J. Rogers (*J. Food Sci.* 49: 573, 1984).

The following are my comments concerning Fig. 1.

1. Since protein molecules with larger molecular weight migrate at a slower rate than those with lower molecular weights in the SDS-PAGE system used by the authors, the last sentence of the Fig. 1 title would be more correct to read "High molecular weight bands corresponding to dimers, trimers and tetramers of myosin heavy chain appear with Rm values of 0.19, 0.11 and 0.08, respectively."

2. It is apparent when comparing peak heights of the various peaks of scan a and b that these two scans were of different protein loads, presumably the protein load of scan b was considerably higher than that of a. Therefore, the comparison of scan a and b may not be a fair one. Ideally, scans of similar protein loads should have been presented for this particular type of comparison.

3. Evidently, the myosin prep used in this experiment contained a fair amount of contaminating actin (peak with Rm between 0.52 and 0.60, scan a). The near complete disappearance of this presumed actin band and the greatly reduced peak height of myosin light chain 1 (LC1) in scan b suggest that the newly formed high molecular weight bands are likely complexes of myosin heavy chain (HC), LC1 and actin with various degrees of polymerization, rather than simply being the dimers, trimers and tetramers of myosin HC as suggested by the authors.

– *Ing C. Peng*, Asst. Professor, Purdue Univ., Smith Hall, West Lafayette, IN 47907.

. . . and the reply

The authors thank Dr. Peng for drawing their attention to the inversion error in the listed Rm values in Fig. 1.

Some reviewers have claimed that densitometer scans should be presented with comparable attenuation to allow ready identification of differences. However, the view that scans of equal protein load should be compared, regardless of peak height, is also valid.

Scope does exist for speculation as to the fate of minor quantities of low molecular weight proteins on the gels. However, the major bands of higher molecular weight than myosin heavy chain appear as discrete bands differing in molecular weight by 200,000 daltons. Thus they correspond to the molecular weight of myosin dimers, trimers and tetramers and it was considered reasonable to state in the text that this was their probable identity. If the bulk of the high molecular weight proteins appeared as a range of heteropolymers, a broader distribution of protein bands with more pronounced smearing would be expected.

– *L. Kurth*, CSIRO Division of Food Research, Meat Research Lab., P.O. Box 12, Cannon Hill, 4170, Queensland, Australia.

– *P.J. Rogers*, School of Science, Griffith Univ., Nathan, 4111, Queensland, Australia.

ERRATA NOTICE

J. Food Sci. (1984) 49(1): 172–176 + 187. Optimization of processing parameters for the preparation of flounder frame protein product by J. Montecalvo Jr., S.M. Constantinides, and C.S.T. Yang. Concerning authorship and attribution, to insure proper credits to all concerned, and to correct the official record, be advised:

1. All requests for reprints should be addressed to Dr. Joseph Montecalvo Jr., Food Science Dept., California Polytechnic State Univ., San Luis Obispo, CA 93407.
2. Research was supported by the Univ. of Rhode Island Agricultural Experiment Station and Dept. of Food Science.
3. Dr. C.S.T. Yang is a member of the Food Science Dept., Univ. of Maine, Orono, ME 04469.

J. Food Sci. (1984) 49(2): 543–546. Air classification and extrusion of navy bean fractions by J.M. Aguilera, E.B. Crisafulli, E.W. Lusas, M.A. Uebersax, and M.E. Zabik. This manuscript was incorrectly labeled "A Research Note" during production and unfortunately was not deleted prior to printing. Please correct accordingly.

J. Food Sci. (1984) 49(3): 668–670 + 684. Characterization of thixotropic behavior of soft cheeses by S. Massaguer-Roig, S.S.H. Rizvi, and F.V. Kosikowski. The unit of viscosity was inadvertantly abbreviated as Pa instead of P for Poise. Please correct accordingly.

J. Food Sci. (1983) 48(6): 1617–1621. Dynamic optimization of dehydration processes: Minimizing browning in dehydration of potatoes by M. Mishkin, I. Saguy, and M. Karel. The authors advise there is an error in Table 1, page 1617, under b_2 parameter (a necessary conversion factor was not included). The correct values for parameter b_2 should be: ≤ 0.15, -7.072; >0.15, -10.24. Please correct accordingly.

J. Food Sci. (1984) 49(3): 876–881. Flow properties of low-pulp concentrated orange juice: Serum viscosity and effect of pulp content by A.A. Vitali and M.A. Rao should have followed, in sequence, Flow properties of low-pulp concentrated orange juice: Effect of temperature and concentration [1984, 49(3): 882–888]. We trust that interested readers have not been confused or inconvenienced in any way by the sequence of these two manuscripts in the Journal.

Corrected.
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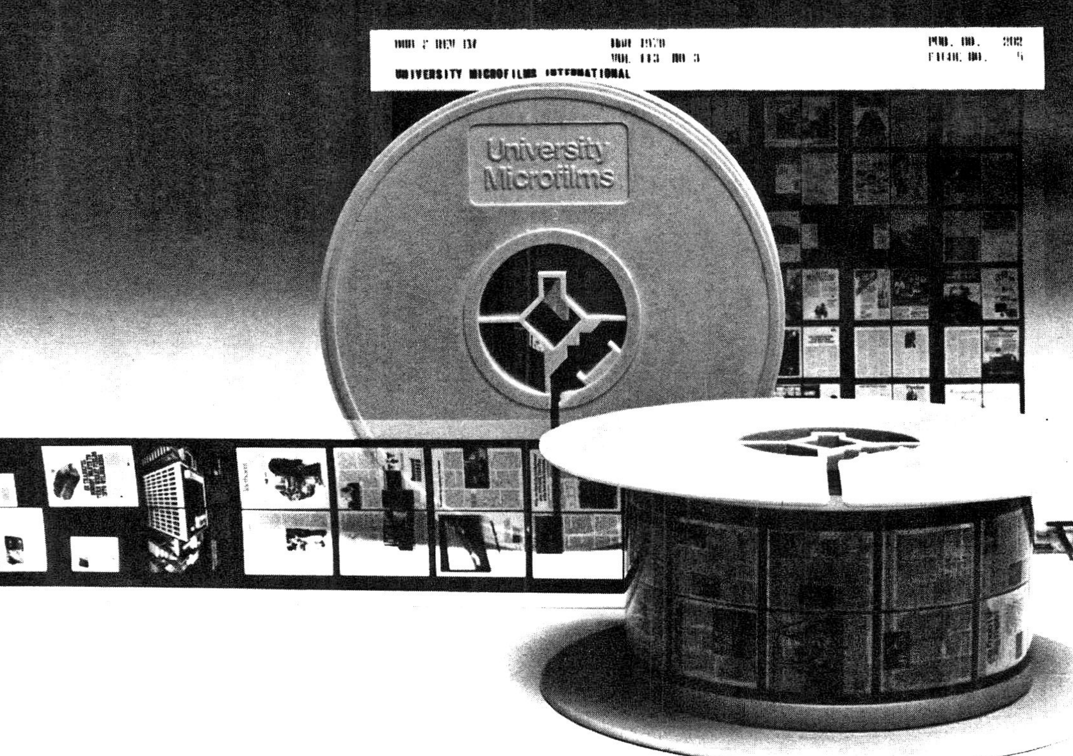

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Evaluation of Methods for Detecting the Production of H_2S, Volatile Sulfides, and Greening by Lactobacilli

B. H. LEE and R. E. SIMARD

ABSTRACT

The ability to produce volatile sulfides by 41 lactobacilli was not associated with species but depended on the composition of the media and the sensitivity of the indicator. Only a restricted number of *L. plantarum* strains and seven isolates were H_2S-positive on peptone iron, TSI and SIM agars. Lead acetate agar detected a greater number of H_2S-positive lactobacilli but the lead acetate (or DTNB) paper method was superior in a modified motility sulfide broth. Cured meat agar detected the production of greening by H_2O_2-producing lactobacilli (*L. fructovorans, L. jensenii, L. lactis, L. viridescens*, F-74, F-99). Such tests, however, were not applicable to detect lactobacilli producing green sulfmyoglobin.

INTRODUCTION

DURING COLD STORAGE of various vacuum- and nitrogen-packaged fresh or cured meats lactobacilli became a predominant part of the psychrotrophs and the extent to which the package remained sensorily sound became a function of lactobacilli numbers (Lee et al., 1983a, b, c; Simard et al., 1983a, b, c). Chilled vacuum-packaged meats after 49 days often gave off objectionable sulfide odors upon opening the pack. Despite the fact that hydrogen sulfide production by lactobacilli is very rare, this form of spoilage in meats has been reported (Shay and Egan, 1981). The production of hydrogen sulfide from cheddar cheese (Kristoffersen and Nelson, 1955; Smith and Cunningham, 1962; Law and Sharpe, 1977) and from peptides (Sharpe and Franklin, 1962) by lactobacilli has also been shown. Sharpe and Franklin (1962) indicated that many strains of lactobacilli can produce hydrogen sulfide under conditions of low pH, anaerobiosis and low carbohydrate concentration. Hanna et al. (1983) also showed that sulfide odors and H_2S are produced in beef steaks, heat sterilized meat and peptone iron agar inoculated with lactobacilli species such as *L. plantarum*, *L. viridescens* and *L. coryneformis*. Shay and Egan (1981) suggested that the ability of lactobacilli to produce hydrogen sulfide from cysteine is a plasmid-mediated character.

Another problem in which color played an important role was greening of meats in vacuum packaging (Lee et al., 1983b, c; Simard et al., 1983c). Green discoloration of cured meats by lactobacilli is due largely to hydrogen peroxide oxidation of meat pigment but sulfmyoglobin greening in fresh meats usually resulted from H_2S produced by *Pseudomonas mephitica* and *Alteromonas putrefaciens* when pH of meat is 6.0 and above (Nicol et al., 1970; Taylor and Shaw, 1977).

However, vacuum-packed fresh meats of low pH (pH 5.8 or lower) also showed a high incidence of greening (Lee et al., 1983b, c; Simard et al., 1983c). Thus the traditional association of greening spoilage of fresh meats with *Pseudomonas* and *Alteromonas* spp. may not be correct if strains of lactobacilli are capable of producing hydrogen sulfide under commonly encountered conditions.

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The objective of this study was to establish the possible role of lactobacilli in sulfide and greening spoilages of packaged meats by evaluating methods for the detection of volatile sulfides and greening. There is also no information available which correlates the production of volatile sulfides and greening by individual strains of lactobacilli.

MATERIALS & METHODS

Bacterial strains and maintenance

The species of *Lactobacillus* used in this study are listed in Table 1. Five strains were obtained from the American Type Culture Collection (ATCC), Rockville, MD. Eight strains were supplied by Dr. R.A. Holley, Food Research Institute, Agriculture Canada, Ottawa, some of which were meat starter isolates. Additional strains were kindly supplied by the Laboratoire de santé publique du Québec. The other isolates were obtained from our culture collection of 50 strains of lactobacilli isolated from spoiled vacuum- or nitrogen-packed fresh or cured meats which had been stored for 49 days at 0-3°C. The numbers of psychrotrophs (composed mainly of lactobacilli) approached 10^8 cells/g at this period and some of the lactobacilli isolated from these products showed greening (g) as well as sulfide odors. Some of the isolates, however, were unable to sustain

Table 1—Strains and sources of lactobacilli used

Strains	Identification no.	Sources
1. *L. plantarum*	ATCC-14917	Pickled cabbage, ATCC
2. *L. plantarum*	ATCC-4008	FRI, Ottawa
3. *L. plantarum*	AN2-251	Human (intestine), LSPQ[a]
4. *L. plantarum*	Lactacel MC	Meat starter, FRI, Ottawa
5. *L. plantarum*	Hansen	Meat starter, FRI, Ottawa
6. *L. casei*	ATCC-334	Cheese, ATCC
7. *L. casei*	ATCC-11443	FRI, Ottawa
8. *L. casei-ss-alactosus*	1700C	Human, LSPQ
9. *L. casei-ss-casei*	AN0-108A	Human, LSPQ
10. *L. casei-ss-rhamnosus*	AN2-265B	Human, LSPQ
11. *L. acidophilus*	AN0-158	Human (Pancreas), LSPQ
12. *L. helveticus*	ATCC-10797	Cheese, ATCC
13. *L. jensenii*	AN2-382	Human (pus), LSPQ
14. *L. lactis*	ATCC-12315	Meat starter, ATCC
15. *L. salivarius*	ATCC-11742	Meat starter, ATCC
16. *L. brevis*	47	Food, LSPQ
17. *L. fermentum*	AN2-255A	Human (intestine), LSPQ
18. *L. fructivorans*	ATCC-8288	Spoiled salad, ATCC
19. *L. viridescens*	ATCC-12706	Sausage, ATCC
20. *Lactobacillus*	Duploferment 66	Meat starter, FRI, Ottawa
21. *Lactobacillus*	Fermentang BN1005	Meat starter, FRI, Ottawa
22. *Lactobacillus*	Lyoflore L110	Meat starter, FRI, Ottawa
23. *Lactobacillus*	R muc	Salami isolate, FRI, Ottawa
24. *Lactobacillus*	Isolate-F74 (g)[b]	Frankfurter, Université Laval
25. *Lactobacillus*	Isolate-F99 (g)	Frankfurter, Université Laval
26. *Lactobacillus*	Isolate-F124	Frankfurter, Université Laval
27. *Lactobacillus*	Isolate-B1	Ground beef, Université Laval
28. *Lactobacillus*	Isolate-B2	Ground beef, Université Laval
29. *Lactobacillus*	Isolate-B3	Ground beef, Université Laval
30. *Lactobacillus*	Isolate-B6 (g)	Ground beef, Université Laval
31. *Lactobacillus*	Isolate-V21	Veal, Université Laval
32. *Lactobacillus*	Isolate-V32	Veal, Université Laval
33. *Lactobacillus*	Isolate-V34	Veal, Université Laval
34. *Lactobacillus*	Isolate-H36	Ham, Université Laval
35. *Lactobacillus*	Isolate-H40	Ham, Université Laval
36. *Lactobacillus*	Isolate-H45 (g)	Ham, Université Laval
37. *Lactobacillus*	Isolate-H48 (g)	Ham, Université Laval
38. *Lactobacillus*	Isolate-P14	Pork, Université Laval
39. *Lactobacillus*	Isolate-P19	Pork, Université Laval
40. *Lactobacillus*	Isolate-24	Pork, Université Laval
41. *Lactobacillus*	Isolate-P161	Pork, Université Laval

[a] LSPQ = Laboratoire de santé publique du Québec
[b] (g) — isolates from green meats

growth on MRS or APT agar on subsequent transfer. All isolates were gram-positive, catalase-negative rods or coccobacilli tested by the methods of Vanderzant and Nickelson (1969). Lyophilized cultures were reconstituted with MRS broth (Difco) at 30°C for 48 hr before inoculation into test media. All cultures were streaked on plates of MRS agar (1.5% w/v), and incubated for 48 hr at 30°C using the Gas-Pak system (Oxoid). All strains were subcultured at 3–4 wk intervals in APT broth (Difco) and maintained at 0°C in MRS agar (0.5%) as stabs and at −30°C in 20% (v/v) glycerol diluted equally with growth medium.

Production of volatile sulfides

Strains were tested for their ability to produce hydrogen sulfide from different media: triple sugar iron agar (TSI, Difco), peptone iron agar (PIA, Difco), sulfide indole motility agar (SIM, Oxoid), lead acetate agar (LAA, Difco) supplemented with 0.1% (v/v) Tween 80 and 0.005% (w/v) $MnSO_4 \cdot 4H_2O$ (Shay and Egan, 1981). Duplicate screw-capped tubes (100 × 13 mm) containing 14 mL of each medium were inoculated by streaking the slant or by stabbing the agar and incubating at 30°C for 2 wk. The reactions were examined daily for blackening of media. The ability to produce hydrogen sulfide, methanethiol or dimethylsulfide from certain amino acids was tested by growing cells in MRS or nutrient broth containing 0.1% (w/v) methionine and 0.02% (w/v) cysteine, (McMeekin et al., 1978), peptone iron broth, a modified motility sulfide broth (MMS, Smith and Cunningham, 1962) containing 0.5% (w/v) tryptone, 0.3% (w/v) yeast extract, 0.3% (w/v) sodium thiosulfate, 0.2% (w/v) sodium acetate, 0.1% (v/v) Tween 80, 0.05% (w/v) glucose and 0.5% (v/v) Rogosa salt solutions (Rogosa et al., 1951). Bacto-casitone medium (BC, Sutherland et al., 1975) containing DL-cystine was also used. Volatile sulfides were detected by placing a sterile filter-paper strip impregnated with a solution of DTNB (5:5′-dithiobis-2-nitrobenzoic acid; McMeekin et al., 1978), or lead acetate in the side-arms of the tubes. The cultures were incubated at 30°C for 10 days. DTNB strips turn bright yellow in the presence of hydrogen sulfide, methanethiol or dimethylsulfide, whereas blackening of lead acetate paper indicates the presence of hydrogen sulfide. Uninoculated controls were prepared in a similar manner.

Analyses of volatile sulfides produced by lactobacilli were carried out by gas chromatography. Except for a capillary column (60m × 0.25 mm i.d.) packed with SE54 and for a carrier gas (H_2, flow rate 1.5 mL/min), the procedure was similar to that of Gardner and Patterson (1975).

Table 2—Detection of volatile sulfide- and greening-producing lactobacilli in various media[a]

Strains	Sulfides		Greening
	LAA	MMS	Meat agar (+nitrite)
1. *L. plantarum*	+	+[w]	−
2. *L. plantarum*	+	+	−
3. *L. plantarum*	+	+	−
4. *L. plantarum*	+	+	−
5. *L. plantarum*	+	−	−
6. *L. casei*	−	+	−
7. *L. casei*	+	+	−
8. *L. casei-ss-alactosus*	−	+	−
9. *L. casei-ss-casei*	−	+	−
10. *L. casei-ss-rhamnosus*	+	+	−
11. *L. acidophilus*	+	+[w]	−
12. *L. helveticus*	+	+[w]	+
13. *L. jensenii*	−	+	+
14. *L. lactis*	+	+[w]	+
15. *L. salivarius*	+	+[w]	−
16. *L. brevis*	+	+	−
17. *L. fermentum*	+	+	−
18. *L. fructivorans*	+	+[w]	+
19. *L. viridescens*	+	+[w]	+[b]
20. *Lactobacillus* (Duploferment)	−	+[w]	−
21. *Lactobacillus* (Fermentang)	+	+[w]	−
22. *Lactobacillus* (Lyoflore)	−	+	−
23. *Lactobacillus* (R muc)	+	+	−
24. *Lactobacillus* (F-74)	+	+[w]	+
25. *Lactobacillus* (F-99)	+	+[w]	+
26. *Lactobacillus* (F-124)	+	+	−
27. *Lactobacillus* (B-1)	+	+	−
28. *Lactobacillus* (B-2)	+	+	−
29. *Lactobacillus* (B-3)	+	+	−
30. *Lactobacillus* (B-6)	+	+	−
31. *Lactobacillus* (V-21)	−	−	−
32. *Lactobacillus* (V-32)	−	+	−
33. *Lactobacillus* (V-34)	−	+	−
34. *Lactobacillus* (H-36)	+	+	−
35. *Lactobacillus* (H-40)	+	+[w]	−
36. *Lactobacillus* (H-45)	+	+	−
37. *Lactobacillus* (H-48)	+	+	−
38. *Lactobacillus* (P-14)	+	+[w]	−
39. *Lactobacillus* (P-19)	−	+[w]	−
40. *Lactobacillus* (P-24)	+	+	−
41. *Lactobacillus* (P-161)	−	+[w]	−

[a] Data from two separate experiments which were run in duplicate; LAAC (lead acetate agar), MMS (modified motility sulfide).
[b] Intensive greening
[w] Weak reaction

Detection of greening

Fresh or cured meat agar was prepared as modified by Shank and Lundquist (1963); 250g of fresh lean beef was blended in a Waring Blendor with 500 mL of molten MRS agar (1.5%). For cured meat agar, 0.075g of sodium nitrite (100 ppm nitrite) was added to the similar preparation of slurry. The slurry was autoclaved for 10 min at 121°C and re-autoclaved for 25 min after the coagulum was blended into a smooth paste. After cooling to 45°C, pour plates were prepared and stored at 4°C. Lactobacilli strains were grown in MRS broth for 24 hr at 30°C and a sterile filter paper disc (¼ in. diam, BBL) was dipped into the culture. Each plate was overlaid with nine culture discs and the discs were pressed slightly to ensure contact. Plates were also streaked with purified colonies without using discs. All plates were incubated for 48 hr at 30°C in an anaerobic jar (Gas-Pak System, Oxoid) and were again incubated aerobically for 24 hr at 30°C. Hydrogen peroxide concentration in MRS culture supernatants was determined by the method described by Price and Lee (1970). A second series of experiments was run to verify the results of volatile sulfides and greening.

RESULTS & DISCUSSION

Detection of volatile sulfide

Among agar media for detecting hydrogen sulfide production by lactobacilli, lead acetate agar detected a greater range of hydrogen sulfide-producers that are potential spoilage lactobacilli (Table 1). Lead acetate agar showed a faint blackening in the presence of most lactobacilli but blackening of lead acetate agar was always strong in *L. plantarum* (ATCC-4008, AN2-251) and isolates (26, 27, 28, 29 and 30). Only a restricted range of strains such as *L. plantarum* (ATCC-4008; AN2-251) and isolates (26, 27, 28, 29, 30, 32, 34) were capable of forming hydrogen sulfide in triple sugar iron, peptone iron and SIM agars. Some isolates (29, 30, 34) showed a negative reaction on TSI. The main difference between TSI and other media appeared to be the presence of sucrose, dextrose and lactose in TSI compared with PIA, LAA (dextrose) and SIM. Further experiments using a more sensitive test with DTNB or lead acetate strips in Bacto-casitone and peptone iron broth did not improve the detection of volatile sulfides. Except for *L. plantarum* AN2-251, *L. casei-ss-rhamnosus*, *L. casei-ss-casei* and isolates (26, 27, 28, 29, 30), poor growth was recorded in these media for the rest of the cultures. When tested with DTNB and lead acetate strips to distinguish between H_2S and other sulfides the results were comparable, suggesting that the sensitivities of two indicators are about the same and most of volatile sulfides produced were hydrogen sulfide. However, blackening with lead acetate strips was more readily detectable than the faint yellow of DTNB strips. A modified motility sulfide medium (Difco) gave superior growth of lactobacilli in liquid culture, which agreed with the results of Smith and Cunningham (1962). This medium, using a lead acetate strip, appeared to be a better substrate for the production of hydrogen sulfide, although blackening of the strips was not heavy and did not occur until the fifth to the seventh day. With the exception of *L. plantarum* (Hansen) and isolate (V-21), most strains of lactobacilli tested were able to produce hydrogen sulfide from this medium, similar to lead acetate agar, but more strains (10)

were positive in MMS medium. This is in agreement with the finding of Sharpe and Franklin (1962) that many lactobacilli produced hydrogen sulfide under appropriate conditions (low pH, anaerobiosis, low sugar). By contrast, all lactobacilli grew very well in nutrient broth or MRS broth containing cysteine and methionine, but none of the lactobacilli strains showed evidence of producing volatile sulfides from these media when tested with DTNB or lead acetate strips. The negative reactions obtained with both DTNB and lead acetate strips indicate that all cultures did not metabolize cysteine and methionine. It is also probable that the acid products of fermentable carbohydrates may suppress the activity of the enzyme which forms hydrogen sulfide (Bulmash and Fulton, 1964) or the genes governing hydrogen sulfide were not expressed in these media. To exclude the possibility of false positive results, gas chromatography analyses of volatile sulfides produced by newly obtained 8 lactobacilli culture (ATCC) were done. Unpublished results confirmed that all of the lactobacilli produced H_2S and other sulfides (CH_3SH and COS) with less intense peaks in MMS medium, although quantitative data are not yet available. It is likely that hydrogen sulfide was produced from thiosulfate by sulfite reductase or from cysteine/cystine by cysteine desulfhydrase (Barker, 1961) and hydrogen sulfide then reacted with free methionine to split the C-S bond and release methanethiol (Manning, 1979).

Detection of greening

In the absence of air (Gas-Pak), greening was not observed during 48 hr incubation at 30°C but when the plates were exposed to air for 24 hr incubation, greening discoloration developed only on cured meat agar (Table 2). Green pigments then faded gradually during further 24 hr incubation. *L. viridescens* always produced more intense greening than *L. fructovorans*, *L. helveticus*, *L. jensenii*, *L. lactis* and isolates (F-74 and F-99).

When the lactobacilli are growing under aerobic conditions, oxygen becomes a hydrogen acceptor and hydrogen peroxide is formed. Greening on cured meat agar is made possible through the inactivation of catalase by heat treatment of by the presence of nitrite. However, hydrogen peroxide detection by the method of Price and Lee (1970) was unsuccessful. Accumulation of hydrogen peroxide in culture medium during growth of lactobacilli was probably not sufficient to be detected in the present experiment. Hydrogen peroxide production was reported to occur in *Lactobacillus* species such as *L. plantarum*, *L. lactis*, *L. bulgaricus*, *L. acidophilus* and *L. viridescens* (Dahiya and Speck, 1968).

All strains tested failed to produce green sulfmyoglobin from uncured meat, which is consistent with the findings that the same strains could not form H_2S in certain media (e.g. nutrient broth + cysteine + methionine). Sharpe and Franklin (1962) however indicated that greening of raw meat by lactobacilli may be caused by activation of H_2S under suitable conditions.

Any attempts to explain why sulfmyoglobin could not be detected, will be speculative until further studies have been performed on many variables such as the amount of growth, the availability of the sulfur source, the oxidation-reduction potential or the presence of fermentable carbohydrates in this media.

So many factors influence the yield of H_2S that a large number of genetic alterations during long term storage of vacuum-packaged raw meats could also affect the amount of H_2S and greening pigments that lead to spoilage of meats.

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หอสมุดกลาง สำนักวิทยาศาสตร์บริการ

Influence of Initial Riboflavin Content on Retention in Pasta During Photodegradation and Cooking

E. M. FURUYA and J. J. WARTHESEN

ABSTRACT

Samples of enriched pasta were stored under controlled lighting conditions and the loss of riboflavin was determined. After an initial rapid degradation, the riboflavin level continued to decrease slowly. After 12 wk, more than 80% the riboflavin had degraded. When pasta samples with varied levels of riboflavin enrichment were exposed to light for 2 days, riboflavin losses ranged from 57.8-64.3%. Riboflavin leaching from pasta during cooking was also shown to be concentration independent with 34.5-37.6% of the riboflavin solubilized in the cooking water. Riboflavin in pasta which had been exposed to light for 2 days prior to cooking had a similar percentage of the undegraded riboflavin leached into the cooking water.

INTRODUCTION

WORK PREVIOUSLY REPORTED from this laboratory (Woodcock et al., 1982) has shown that riboflavin in enriched pasta undergoes photodegradation in two phases with each phase proceeding by a first order mechanism. This is to be distinguished from the single phase first order mechanism that describes the photodegradation of riboflavin in fluid milk (Allen and Parks, 1979). While a two-phased mechanism may best describe riboflavin degradation in pasta, a detailed and expanded evaluation of riboflavin degradation in pasta exposed to light would provide a better understanding of the stability of this vitamin. Quantifying riboflavin photodegradation in enriched pasta starting with several different riboflavin levels presents an alternative approach for determining if the degradation is first order. Determining riboflavin retention in pasta at regular intervals through several months of light exposure would indicate if the rate of riboflavin degradation is constant after extensive degradation has already taken place.

As a water-soluble vitamin, riboflavin can leach from pasta into the cooking water; thus, leaching during preparation is another major mode of riboflavin loss. Dexter et al. (1982) reported only 45-52% of the original riboflavin was retained by the spaghetti during cooking, while Abdel-Rahman (1982) reported 55% retention after cooking for 15 min. One possible source of variation in riboflavin retention in cooked spaghetti is the variable enrichment levels of riboflavin in the uncooked pasta. The enrichment level range is from 3.8–4.9 μg riboflavin/g pasta in the United States (Code of Federal Regulations, 1979) and from 6.5-19.5 μg/g in Canada (Dexter et al., 1982). Additional study may help to clarify the extent of riboflavin leaching during cooking and the impact of the level of enrichment on riboflavin retention.

The photodegradation of riboflavin in pasta prior to cooking could influence the proportion of riboflavin retained in cooked spaghetti. By storing spaghetti in transparent containers or packages, an appreciable amount of riboflavin may be degraded before the cooking process begins. Analysis of riboflavin retained in spaghetti which had been exposed to light before cooking would indicate the extent of additional riboflavin loss with cooking.

The objectives of this study were to evaluate riboflavin photodegradation in pasta by monitoring the change in concentration over short time intervals and over a period of three months. Riboflavin loss was also evaluated by quantifying riboflavin photodegradation in pasta with varied enrichment levels to determine if the reaction was first order. The leaching of riboflavin during the cooking of pasta was also determined using pasta with several different riboflavin enrichment levels and pasta which had been exposed to light prior to cooking.

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MATERIALS & METHODS

Controlled light exposure

Chambers maintained at a constant light intensity of 150 ft-c, a relative humidity of 44% and room temperature were used in studies where light exposure conditions were controlled. Constant light intensity was regulated with variable intensity standard fluorescent lights (Cool White, General Electric No. F15T8-CW) and was monitored with a General Electric Type 214 light meter. Saturated K_2CO_3 was used to provide a constant relative humidity of 44% in the chambers.

Evaluation of photodegradation

To appraise the degradative behavior of riboflavin in pasta, enriched elbow macaroni was obtained fresh from a local processor and protected from light during handling. A single layer of this elbow macaroni was exposed to light and duplicate samples were taken every 3 hr from 0-24 hr, every 6 hr through 48 hr, every 12 hr through 4 days and then once a day through 7 days. Samples were also taken once a week for 12 wk.

Evaluation of riboflavin photodegradation with varied initial concentrations of riboflavin enrichment was accomplished by exposing single layers of spaghetti to light. Spaghetti with five different levels of riboflavin were obtained by surveying commercially available products in the Minneapolis/St. Paul area, as well as Fort Francis, Ontario. Ten samples at each level of enrichment were exposed to light and five replicates of each were analyzed after 2 and 7 days of exposure. Initial riboflavin contents in the spaghetti were 16.36 (A), 10.77 (B), 6.58 (C), 5.24 (D) and 3.75 (E) μg/g spaghetti. The samples were coded as indicated by the letters in parentheses. Only products that were packaged in paperboard cartons with no detectable lumichrome were used. Lumichrome is a known photodegradation product of riboflavin and has been shown to occur in pasta after light exposure. (Woodcock et al., 1982). The mean retention values were compared using an F test followed by a least significant difference test with $P = 0.05$ (Steel and Torrie, 1980).

Leaching with cooking

Three of the five spaghetti samples discussed above (A, D and E) were used to determine the extent of leaching before and after photodegradation of riboflavin. After exposing a single layer of spaghetti to a light with an intensity of 150 ft-c for 2 days, the exposed and unexposed samples were cooked. Spaghetti was prepared according to the package directions by placing 50g spaghetti in 500 mL rapidly boiling water for 10 min. After cooking and rinsing with 20 mL water, the spaghetti was drained for 3 min. The draining and cooking water were combined, the volume was measured, and the water was analyzed for riboflavin. The cooked weight of spaghetti, which is a measure of the water absorbing characteristics, was

determined to be about 2.7 times the dry weight of the spaghetti, indicating that normal water absorbance and complete cooking had occurred (Vasiljevic and Banasik, 1980).

The cooked spaghetti was frozen and then lyophilized for 18 hr. Preliminary tests showed that 18 hr was sufficient to reach constant weight. After freeze-drying, the samples were ground and weighed out for immediate vacuum oven moisture analysis (AOAC, 1980) and riboflavin analysis. Moisture and riboflavin analyses were done on the uncooked samples as well, and the results were reported as apparent retention (Murphy et al., 1975).

$$\text{Apparent retention} = \frac{\mu\text{g riboflavin/g cooked pasta (dry basis)}}{\mu\text{g riboflavin/g uncooked pasta (dry basis)}} \times 100$$

All cooking and handling procedures were conducted in a darkened room. Five replicate samples were cooked and analyzed in duplicate for each treatment.

Quantification of riboflavin

Extraction of riboflavin from uncooked elbow macaroni, uncooked spaghetti, and freeze-dried spaghetti was accomplished using a modified AOAC (1980) riboflavin procedure. Samples were ground in a micromill to a particle size sufficiently small enough to pass through a No. 20 sieve. Samples weighing 1.50g were transferred into capped Pyrex test tubes that were wrapped in aluminum foil to prevent light exposure of the samples during handling. Fifteen mL 0.1N HCl was added, the samples were mixed, and then autoclaved for 30 min at 15 psi. The samples were remixed, centrifuged at 270 x *g* for 30 min, and the supernatants were decanted into 25 mL volumetric flasks. The sediments were rinsed with 10 mL 0.1N HCl, centrifuged and decanted. The extracts were brought to volume using 0.1N HCl. The extracts were filtered through 0.45 μm membrane filters, and riboflavin was quantified by high performance liquid chromatography (HPLC). The HPLC system consisted of a Model 6000A pump (Waters Associates, Inc.), a Model 7120 Rheodyne injector with a 10 μL sample loop, a Model FS Fluoromat fluorometer (Kratos, Inc.) and a Hewlett Packard Model 3380A recorder integrator. The separation of riboflavin was accomplished using a RP-8 column (10 micron, 200 mm x 4.6 mm, Hewlett Packard) with a mobile phase of 43% methanol, 56% distilled water and 1% glacial acetic acid at a flow of 1.0 mL/min. A 7-59 excitation filter and a 3-70 emission filter were used for the fluorescence detection of riboflavin.

Riboflavin was quantified by comparing the peak heights of the samples to the peak heights of standard riboflavin solutions. Standard solutions were prepared by dissolving riboflavin in 0.1N HCl, using heat and stirring, and then serially diluting to a concentration of 0.15 μg riboflavin/mL.

The absence of lumichrome in pasta samples was established by the absence of a peak on the chromatogram at the expected lumichrome elution time of 5.5 min. Lumichrome elution time was established by injection of a standard lumichrome solution (Aldrich Chemical Co.). This solution was prepared in methanol and then serially diluted with 0.1N HCl to a concentration of 0.30 μg lumichrome/mL. For lumichrome analysis by HPLC, a mobile phase of 49% methanol, 50% distilled water and 1% acetic acid was used at 1.5 mL/min with a FSA 403 excitation filter and a FSA 426 emission filter.

RESULTS & DISCUSSION

Evaluation of photodegradation

When elbow macaroni was exposed to a light intensity of 150 ft-c at room temperature, approximately 50% of the riboflavin photodegraded after only 3 hr (Fig. 1). This rapid rate of riboflavin loss is comparable to riboflavin photodegradation rates in acetone, ethanol or dioxane solutions where approximately 70% of the riboflavin was reported to be photodegraded after 3 hr of light exposure (Koziol and Knobloch, 1965). In contrast, when riboflavin solutions in water were exposed to light for 3 hr, approximately 10% of the riboflavin was degraded. Analysis of riboflavin's electronic excitation in ethanol, acetone and dioxane relative to water illustrated that these solvents caused a shift in the fluorescence maxima toward the ultraviolet wavelengths and were responsible for the decrease in riboflavin stability (Koziol and Knobloch, 1965). The speed of the initial riboflavin photodegradation when pasta is exposed to light indicates the riboflavin in pasta may be in a chemical environment similar to that of riboflavin in an organic solvent and therefore it readily degrades with light exposure.

After an initial rapid loss, the photodegradation of riboflavin was shown to gradually decrease and then to occur much more slowly over 3 months (Fig. 2). When the riboflavin levels are considered from week 1 through week 12, the riboflavin decreased linearly from approximately 1.6 to 1.0 μg/g pasta. This slow rate of loss may be due to light penetration limitations. Macaroni packaged in transparent material and exposed to light would likely show extensive loss of riboflavin in the outer layers but relatively little riboflavin degradation in layers away from the surface.

Results from the study in which spaghetti samples with five different initial riboflavin contents were exposed to light showed higher levels of riboflavin remaining in samples with higher enrichment levels (Table 1). The mean riboflavin content in the control (unexposed) samples ranged from

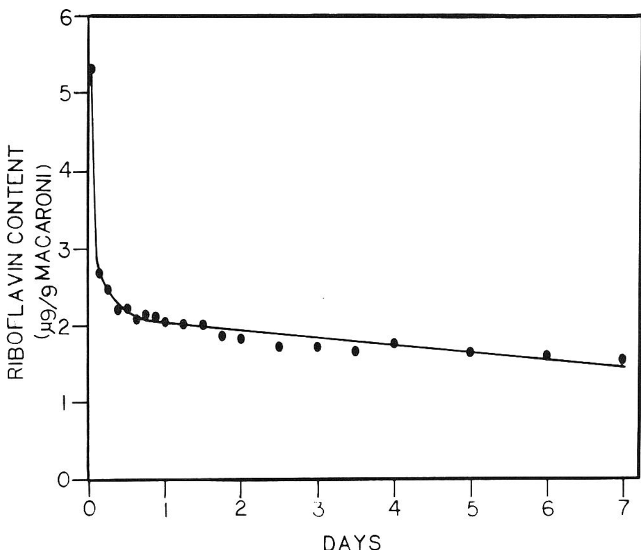

Fig. 1–Riboflavin content in elbow macaroni exposed to light with frequent sampling.

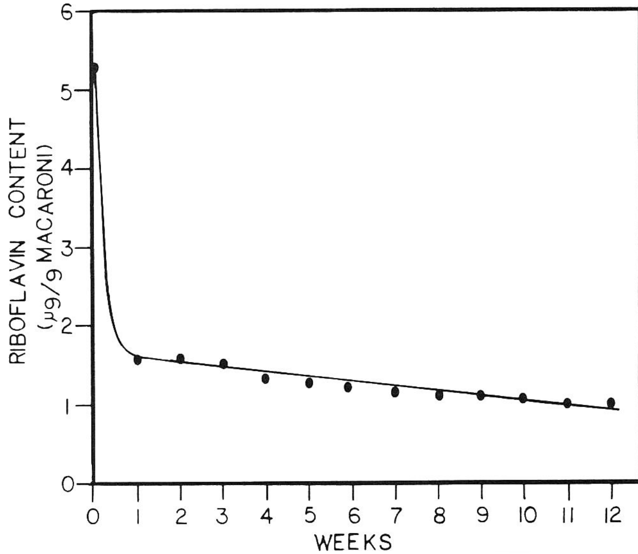

Fig. 2–Riboflavin content in elbow macaroni exposed to light with sampling over an extended period of time.

Table 1—Riboflavin content in spaghetti exposed to light for 0, 2, and 7 days

	Riboflavin content (μg/g pasta)[a]		
Sample	0 days	2 days	7 days
A	16.36 ± 0.29	6.69 ± 0.12	6.31 ± 0.21
B	10.77 ± 0.21	3.84 ± 0.16	2.94 ± 0.14
C	6.58 ± 0.28	2.78 ± 0.12	2.04 ± 0.17[b]
D	5.24 ± 0.16	2.17 ± 0.09	1.87 ± 0.07[b]
E	3.75 ± 0.09	1.35 ± 0.13	1.00 ± 0.04

[a] Average of 5 determinations ± standard deviations.
[b] Means are not significantly different at P = 0.05.

Table 2—Ordered percent retention of riboflavin in spaghetti exposed to light for 2 and 7 days

2 days		7 days	
Sample	% Retention	Sample	% Retention
C	42.2[a]	A	38.6[c]
D	41.4[a]	D	35.7[c]
A	40.9[a]	C	31.0[d]
E	36.0[b]	B	27.3[d]
B	35.7[b]	E	26.7[d]

[a-d] Retentions with the same letter are not significantly different within each column. Each 7-day sample was significantly lower than the corresponding 2-day sample.

Table 3—Riboflavin retention and loss into the cooking water for spaghetti at varied initial riboflavin levels and for spaghetti exposed to light[a]

	Unexposed to light			Exposed to light		
Sample	Uncooked riboflavin content (μg/g spaghetti)	Retention in cooked spaghetti (%)	Leached into cooking water (%)	Uncooked riboflavin content (μg/g spaghetti)	Retention in cooked spaghetti (%)	Leached into cooking water (%)
A	16.36	56.7 ± 3.0	34.5 ± 1.3	6.69	61.9 ± 3.7	34.3 ± 1.4
D	5.24	56.7 ± 3.8	37.6 ± 1.7	2.17	59.6 ± 6.0	33.2 ± 0.6
E	3.75	59.3 ± 2.7	37.5 ± 0.9	1.35	64.5 ± 3.8	32.1 ± 2.6

[a] Retention is reported as mean ± standard deviation

3.75-16.36 μg/g pasta. After 2 days the riboflavin contents ranged from 1.35-6.69 μg/g pasta, and after 7 days contents ranged from 1.00-6.31 μg/g pasta. All of the mean riboflavin contents were significantly different from each other except the levels found in samples C and D after 7 days.

When the data in Table 1 are converted to percent retention and ordered by magnitude (Table 2) it appears that the initial riboflavin content did not have a great influence on the percent retention with light exposure. Some significant differences between mean percent riboflavin retentions were observed, however, the overall difference in percent retention for the five samples was relatively small. After 2 days the retention ranged from 35.7-42.2%, and after 7 days the range was 26.7-38.6%. Varying the concentration of the reactant over a broad range and observing a constant rate of degradation or a constant half-life provide kinetic evidence that the reaction is first order. Most foods have a relatively small range of nutrient contents, but because pasta may be fortified to varied levels in the United States and Canada, this type of kinetic determination is possible. While the difference in riboflavin concentration in the enriched spaghetti samples used was about fourfold, the narrow range of percent retention after 2 days of light exposure confirmed the work of Woodcock et al. (1982) showing that the rapid degradation of riboflavin in pasta is first order. Previous work had only used rate constants and correlation coefficients to determine reaction order. The percent retention after 7 days was representative of degradation that was well into the second (slower) phase. The decrease in percent retention between day 2 and day 7 averaged 7.5% across all samples. However, the retention in sample A was only 2% less with the additional 5 days of exposure, while sample C decreased by 11%. After 7 days of exposure and extensive degradation, there may be a small effect of spaghetti brand on riboflavin retention but no apparent effect of initial enrichment level on percent retention.

The percentage of riboflavin degraded in spaghetti samples with five different enrichment levels was relatively constant; these results indicate that an increase in the enrichment level would increase the riboflavin content in consumer pasta products, even if photodegradation of riboflavin occurs.

Leaching with cooking

Table 3 shows that the retention of riboflavin in cooked samples not previously exposed to light was relatively constant regardless of the initial riboflavin content. Riboflavin retentions of 56.7%, 56.7% and 59.3% were observed with no significant differences among the amounts of riboflavin retained in the samples.

Spaghetti corresponding to the same samples discussed above were exposed to light for 2 days and then cooked. These samples showed retentions of 59.6%, 61.9% and 64.5% when retention for the exposed-cooked spaghetti was calculated from the ratio of riboflavin in the cooked exposed spaghetti (dry basis) to the riboflavin in the uncooked exposed spaghetti (dry basis). Evaluation of the mean retention values for cooking after light exposure indicated there was no significant difference in percent retention among the three exposed samples and there was no significant difference in percent cooking retention between the exposed and unexposed samples.

The riboflavin found in the cooking water accounted for most of the riboflavin not retained in the spaghetti. Total recovery from the sum of the riboflavin in the cooking water and in the cooked spaghetti was approximately 95%. Dexter et al. (1982) reported 45 - 52% riboflavin retention with cooking and a total recovery of only 60%. Since riboflavin has been reported to be thermally stable during short periods of heating (Kearsley and Rodriguez, 1981), photodegradation of riboflavin during cooking and freeze drying may have been responsible for the low observed recovery.

Dexter et al. (1982) also considered leaching of nutrients in spaghetti using two enrichment levels. A weak interaction between enrichment level and riboflavin leaching was observed. However, nonconventional, high temperature drying techniques used in the preparation of the spaghetti may have affected riboflavin leaching.

The cooking conditions were constant throughout the experiments discussed, although a change in the volume of cooking water or cooking time could affect retention of riboflavin in cooked spaghetti. These results show that riboflavin retention in cooked spaghetti was in the range of 56.7 - 64.5% and did not appear to be strongly influenced

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Characteristics of Israeli Citrus Peel and Citrus Juice

E. COHEN, R. SHARON, L. VOLMAN, R. HOENIG, and I. SAGUY

ABSTRACT

Data on the composition of Israeli orange juice, grapefruit juice, orange peel and grapefruit peel were analyzed. Significant differences were found mainly between the juice and peel in the content of isocitric acid, ash, minerals, total pectin, total flavonoids, chlorides, phosphates, chloramine-T number and arginine. The significance of these findings in detecting juice adulteration with peel extract solids is discussed. The chemical and amino acids characteristics of the citrus products analyzed may be used to establish guidelines required for detecting citrus juice adulteration.

INTRODUCTION

IN RECENT YEARS there has been world wide interest in methods for detecting adulteration of citrus products, mainly citrus juice and concentrate. Adulteration can range from a simple addition of sugar solution to more sophisticated methods, such as addition of pulpwash solids or peel extract solids.

The composition of citrus juice (Cohen et al., 1983a, b; Petrus and Vandercook, 1980; Wallrauch, 1980, 1981a, b) and methods for detecting adulteration and verifying the authenticity of citrus juice (Brown and Cohen, 1983; Lifshitz et al., 1974; Petrus and Attaway, 1980; Schatzki and Vandercook, 1978; Vandercook et al., 1983) were widely documented. Most of the methods suggested for detecting adulteration of citrus juice are based on comparing characteristic values of an unknown sample with those of pure juice. However, there is normally large variability in natural fruit juice attributes due to growing season, geographical origin, environmental and agrotechnical factors (Brown and Cohen, 1983; Brown et al., 1981; Cohen, 1982; Park et al., 1983; Petrus and Vandercook, 1980; Wallrauch, 1980). This variability emphasizes the need for a wide base sample which would be useful in sensitive discriminant statistical analysis required for determinations of the purity of fruit juice (Brown and Cohen, 1983).

Citrus peel solids are a natural source which could be used in juice adulteration. Benk (1968) reported adulteration of citrus juices with rag and peel extracts and noted that detecting such adulteration should be quite easy, as pentose equivalent values are about three times higher than those obtained for orange juice. Sawyer (1963) included ascorbic acid and nicotinic acid in order to detect orange juice adulteration by dilution or addition of peel extract. Park et al. (1983) and Vandercook et al. (1983) provided a broad base of compositional data on California Navel orange juice. This work was undertaken to provide data on Israeli citrus juice and citrus peel composition.

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MATERIALS & METHODS

Sampling

Samples of citrus juice (orange and grapefruit) and citrus peel were taken by official inspectors, directly from the extraction machines (FMC, in-line juice extractor) in citrus plants located throughout Israel. The samples were taken at random, over the entire processing season (Oct. 1981 through June 1982). The samples were frozen or pasteurized, and analyzed periodically.

Analytical methods

Samples of citrus peel (ca 300g) were cut into pieces approximately 1x1 cm^2, mixed with distilled water (1:1, w/w), crushed and blended. The resultant mixtures were analyzed. Citrus juice were analyzed without further treatment.

All the characteristics were analyzed according to standard methods used for quality control of citrus juice (Anon., 1982). The methods are summarized as follows:

Total soluble solids. AOAC (1980) method #31.011; Total sugars after inversion: AOAC (1980) methods #31.038, 31.042 and 52.019; Acidity: AOAC (1980) method #22.060; Isocitric and malic acid: Enzymatic method (Boehringer and Mannheim, 1980); Ash: AOAC (1980) method #22.026; Calcium and magnesium: AOAC (1980) methods #22.044, 2.019, 2.110 and 2.112; Chlorides: Potentiometric measurement using a silver electrode (Best, 1950); Phosphorus: AOAC (1980) methods #22.042–22.045; Sulphates: AOAC (1980) methods #33.120–33.123; Potassium and sodium: The samples were filtered through Whatman #41 paper (ashless) and the transmittance at 769 nm and 589 nm were determined using an emission flame photometer (Varian Model 1000).

Formol number. 10 mL of sample was adjusted to pH 8.3 with 0.1N NaOH. The acidity liberated by the addition of 10 mL 40% formaldehyde was determined by titration to pH 8.3 with 0.1N NaOH.

Chloramine T number. Titration with 0.01N $Na_2S_2O_3$ of iodine liberated by 25 mL 0.01N chloroamine T solution in the presence of KI crystals added to 1 mL of sample.

Total flavonoids. Overnight extraction of flavonoids with alkaline methanol, filtration, and colorimetric determination at 420 nm (Davis, 1947).

Total pectin. Total pectic substances were precipitated by ethanol and redissolved in an alkaline solution. The resulting galacturonic acid was determined by carbazole reaction in the presence of sulfuric acid. The red color produced was measured at 525 nm (Anon., 1964; Bitter and Muir, 1962).

Free amino acids. Samples were centrifuged after celite addition. An aliquot was used for amino acid analyses (Technicon automatic amino acid analyzer, TSM; 0.1 mmol/L sensitivity).

For comparison purposes, all results are given on a weight basis.

Statistical analyses

Statistical analyses were carried out using a computer-aided statistical software (Dixon, 1981).

RESULTS & DISCUSSION

TO DIFFERENTIATE between natural citrus juice and other jucies which may contain peel extract solids, several important characteristics expressing the composition of Israeli single-strength citrus juice and citrus peel are presented in Tables 1 through 4. These tables include the mean value ($\bar{x}$) standard deviation (SD), coefficient of variation (CV = SD/$\bar{x}$), limits (95% confidence interval) and the range of the samples analyzed.

Comparison of citrus peel and citrus juice

Carbohydrates. Total sugars after inversion in both orange and grapefruit juice and peel were not found to be significantly different (Tables 1 and 3). Differentiation between specific sugars (i.e., glucose, fructose, sucrose) is not of interest, as their concentration depends on the processing and/or storage conditions (Wallrauch, 1980).

Acids. Total acidity in orange peel and orange juice (Table 1) was quite similar. No such similarity was found in grapefruit products (Table 3). Isocitric acid was different in citrus juice and peel (15.0-63.5 and 64-159 mg/kg in orange peel and orange juice, respectively, Table 1; and 14.4-61.6 and 125-237 mg/kg in grapefruit peel and grapefruit juice, respectively, Table 3). The wide difference in the isocitric acid content in citrus peel and citrus juice makes this characteristic very valuable for the detection of the addition of peel extract to natural citrus juice. A similar phenomenon was found for the ratio of acidity/isocitric acid

Table 1—Comparison between Israeli orange peel and orange juice: chemical characteristics

		Orange peel (n = 33)[a]						Orange juice (n = 78)[a]			
						Range					
Characteristics	Units	Mean	SD[b]	CV, %[c]	Limits[d]	Min	Max	Mean	SD[b]	CV, %[c]	Limits[d]
Total soluble solids	°Bx	12.5	1.95	15.6	8.6 - 16.4	9.4	17.0	12.0	0.6	5.0	10.8 - 18.2
Total sugars after inversion	% w/w	8.0	1.25	15.6	5.5 - 10.5	5.7	9.8	9.2	0.5	5.5	8.2 - 10.3
Acidity, as citric acid anhydrous	% w/w	0.94	0.12	12.8	0.71 - 1.17	0.74	1.17	1.13	0.2	16.9	0.75 - 1.52
Isocitric acid	mg/kg	32.1	8.6	26.8	15.0 - 63.5	21.0	59.0	112	23.5	21.0	64 - 159
L-Malic acid	g/kg	0.34	0.14	41.2	0.05 - 0.63	0.13	0.67	0.96	0.29	30.0	0.38 - 1.54
Ash	% w/w	0.57	0.12	21.9	0.32 - 0.82	0.44	0.90	0.29	0.04	12.2	0.22 - 0.37
Potassium	mg/kg	940	189	20.1	562 - 1318	590	1440	1373	201	14.6	972 - 1774
Sodium	mg/kg	30			5 - 60	5	90	8.0			- 20
Calcium	mg/kg	1364	402	29.5	560 - 2170	860	2520	94	16	17.0	62 - 127
Magnesium	mg/kg	139	36	25.9	67 - 211	81	252	86	8.5	9.9	69 - 103
Chlorides	mg/kg	159	34	21.4	91 - 227	98	228	84	13.5	16.0	57 - 111
Phosphates (PO_4^{-3})	mg/kg	506	55	10.9	392 - 616	392	650	490	67	13.7	356 - 625
Sulfates ($SO_4^=$)	mg/kg	277	84	30.3	110 - 444	140	480	147	28	19.0	91 - 203
Formol No.	mL NaOH 0.1 N/100g	27.5	4.6	16.7	18.3 - 36.7	18.4	37.0	24.0	3.1	12.9	17.9 - 30.1
Chloramine No.	mL chloramine-T 0.01 N/g	17.3	1.2	6.9	14.9 - 19.7	15.6	29.4	12.0	1.06	8.8	9.6 - 13.9
Total flavonoids * (as hesperidine)	g/kg	18.5	2.5	13.5	13.45 - 23.6	14.6	23.9	1.38	0.28	20.4	0.82 - 1.94
Total pectin	g/kg	3.65	1.50	41.1	0.65 - 6.62	1.7	7.3	0.56	0.13	23.2	0.30 - 0.81
Acidity/Isocitric acid		310	70	22.6	170 - 450	170	460	102	11	10.8	80 - 124
Potassium/ash	%	16.6	2.3	13.9	12.0 - 21.3	12.7	21.2	45.1	3.1	6.8	39.0 - 51.3
Calcium/ash	%	23.8	2.6	10.9	18.6 - 29.0	18.8	28.6	3.2	0.5	15.4	2.2 - 4.4
Magnesium/ash	%	2.4	0.3	12.5	1.8 - 3.1	1.8	2.9	2.9	0.26	9.0	2.4 - 3.5
Phosphates/ash	%	9.1	1.4	15.4	6.3 - 11.9	6.3	11.9	16.8	2.1	12.5	12.3 - 21.2
Formol/proline (g/L)		17.3	3.0	17.3	11.3 - 23.3	12.4	24.9	19.0	3.3	17.4	12.4 - 25.6

[a] n = number of samples
[b] Standard deviation
[c] Coefficient of variation
[d] 95% confidence interval

Table 2—Comparison between Israeli orange peel and orange juice: amino acids content

	Orange peel (n = 33)[a]						Orange juice (n = 78)[a]			
					Range					
Amino acids, mmol/kg	Mean	SD[b]	CV, %[c]	Limits[d]	Min	Max	Mean	SD[b]	CV, %[c]	Limits[d]
Lysine	0.48	0.12	25.0	0.23 - 0.73	0.28	0.74	0.35	0.12	34.3	0.10 - 0.60
Arginine	1.79	0.57	31.8	0.80 - 3.05	0.70	3.28	4.00	1.08	27.0	2.09 - 6.32
Aspartic acid	1.41	0.39	27.7	0.64 - 2.18	0.58	2.16	2.45	0.37	15.1	1.73 - 3.17
Serine + threonine + asparagine	5.86	2.45	41.8	0.96 - 10.76	1.72	10.84	4.48	0.88	19.6	2.73 - 6.23
Glutamic acid	0.32	0.17	53.1	traces - 0.71	0.06	0.74	0.94	0.16	17.0	0.62 - 1.27
Proline	14.17	2.85	20.1	8.47 - 19.87	7.46	20.10	11.38	2.64	23.2	6.09 - 16.66
Glycine	0.37	0.09	24.3	0.20 - 0.55	0.14	0.54	0.27	0.08	29.6	0.13 - 0.42
Alanine	1.37	0.48	35.0	0.41 - 2.32	0.80	2.96	1.40	0.45	32.1	0.50 - 2.31
Valine	0.20	0.08	40.0	traces - 0.32	traces	0.32	0.19	0.06	31.6	traces - 0.32
Isoleucine	0.07	0.07	100.0	traces - 0.26	traces	0.26	0.07	0.05	71.4	traces - 0.23
Leucine	0.19	0.13	68.4	traces - 0.48	traces	0.48	0.05	0.04	80.0	traces - 0.18
Tyrosine	0.04	0.06	150.0	traces - 0.22	traces	0.22	0.04	0.04	100.0	traces - 0.15
Phenylalanine	0.09	0.09	100.0	traces - 0.26	traces	0.44	0.09	0.10	111.0	traces - 0.31
γ-aminobutyric acid	3.06	0.79	25.8	1.68 - 4.75	2.00	5.12	3.30	1.09	33.0	1.12 - 5.47

[a] n = number of samples
[b] Standard deviation
[c] Coefficient of variation
[d] 95% confidence interval

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(Tables 1 and 3). The concentration and the ratio of acidity/isocitric acid and citrate are in agreement with data reported previously (Petrus and Vandercook, 1980), suggesting possible applicability of isocitric acid determination in detection of adulteration.

Malic acid content was not significantly different between citrus juice and citrus peel. The isocitric acid and malic acid data of Israeli orange juice are in agreement with the corresponding values found for California Navel orange juice (Park et al., 1983).

Minerals. The ash content of citrus peel was much higher than that of citrus juice. The high concentration of calcium in the peel was the main reason for this significant difference (Tables 1 and 3). This finding is in agreement with Royo-Iranzo and Gimenez-Garcia (1974), who suggested using the serum to detect adulteration. They reported that sodium, calcium and phosphorus were higher in the pulp than in the serum. Park et al. (1983) indicated that the high content of potassium and calcium in pulpwash must reflect some leaching of potassium and calcium from the pulp. They

Table 3—Comparison between Israeli grapefruit peel and grapefruit juice: chemical characteristics

		Grapefruit peel (n = 21)[a]						Grapefruit juice (n = 48)[a]			
						Range					
Characteristics	Units	Mean	SD[b]	CV, %[c]	Limits[d]	Min	Max	Mean	SD[b]	CV, %[c]	Limits[d]
Total soluble solids	°Bx	10.9	1.25	11.5	8.4 - 13.4	8.6	14.0	10.6	0.7	6.6	9.2 - 12.0
Total sugars after inversion	% w/w	6.7	0.9	13.4	4.9 - 8.5	3.9	7.9	7.5	0.5	6.5	6.5 - 8.5
Acidity, as citric acid anhydrous	% w/w	0.93	0.12	12.9	0.68 - 1.18	0.72	1.14	1.63	2.8	17.2	1.07 - 2.18
Isocitric acid	mg/kg	38.0	11.8	31.0	14.4 - 61.6	23.0	64.0	181.0	28	15.4	125 - 237
L-Malic acid	g/kg	0.43	0.16	37.2	0.11 - 0.75	0.19	0.82	0.42	0.13	31.0	0.16 - 0.71
Ash	% w/w	0.57	0.07	12.3	0.43 - 0.71	0.49	0.73	0.25	0.03	11.7	0.19 - 0.32
Potassium	mg/kg	1077	131	12.2	815 - 1340	830	1290	1145	109	9.5	928 - 1363
Sodium	mg/kg	38	14	36.8	10 - 66	20	70	8.9	13.5	152	- 30
Calcium	mg/kg	1315	214	16.3	887 - 1743	970	1740	93	17	18.3	60 - 126
Magnesium	mg/kg	134	24	17.9	86 - 182	108	196	83	9	10.8	64 - 101
Chlorides	mg/kg	152	40	26.3	72 - 232	92	242	65	13	20.1	39 - 91
Phosphates (PO_4^{-3})	mg/kg	414	64	15.5	285 - 543	227	487	405	34	8.4	337 - 473
Sulfates ($SO_4^{=}$)	mg/kg	227	39	17.2	149 - 305	160	290	129	19	14.7	91 - 167
Formol No.	mL NaOH 0.1 N/100g	22.8	5.4	23.7	12.0 - 33.6	15.0	31.2	22.4	2.2	9.8	18.0 - 26.8
Chloramine No.	mL chloramine-T 0.01 N/g	23.8	7.5	31.5	8.8 - 38.8	18.3	39.0	13.2	0.87	6.6	11.4 - 14.9
Total flavonoids (as naringine)	g/kg	12.3	2.5	20.3	7.3 - 17.3	7.2	18.1	0.80	0.14	17.5	0.50 - 1.09
Total pectin	g/kg	2.95	1.34	45.4	0.27 - 5.63	1.22	5.84	0.43	0.11	25.6	0.20 - 0.64
Acidity/Isocitric acid		263	74	28.1	115 - 411	174	391	89	11	12.4	67 - 111
Potassium/ash	%	19.2	2.6	13.5	14.0 - 24.4	15.1	23.7	46.5	2.6	5.6	41.3 - 51.8
Calcium/ash	%	22.9	2.5	10.9	17.9 - 27.9	19.4	25.7	3.8	0.9	23.6	2.1 - 5.5
Magnesium/ash	%	2.3	0.3	13.0	1.7 - 2.9	1.6	2.9	3.3	0.3	9.3	2.7 - 3.9
Phosphates/ash	%	7.3	1.5	20.5	4.3 - 10.3	3.3	9.6	16.3	2.2	13.6	11.9 - 20.8
Formol/proline (g/L)		21.9	4.2	19.2	13.5 - 30.3	15.1	31.6	34.7	8.8	25.4	17.1 - 52.3

[a] n = number of samples
[b] Standard deviation
[c] Coefficient of variation
[d] 95% confidence interval

Table 4—Comparison between Israeli grapefruit peel and grapefruit juice: amino acids content

	Grapefruit peel (n = 21)[a]						Grapefruit juice (n = 48)[a]			
					Range					
Amino acids, mmol/kg	Mean	SD[b]	CV, %[c]	Limits[d]	Min	Max	Mean	SD[b]	CV, %[c]	Limits[d]
Lysine	0.39	0.13	33.3	0.13 - 0.65	0.24	0.74	0.25	0.08	32.0	0.10 - 0.40
Arginine	1.11	0.46	41.4	0.19 - 2.03	0.20	2.02	2.64	0.72	27.3	1.38 - 4.19
Aspartic acid	2.57	0.77	30.0	1.03 - 4.11	1.30	4.70	5.10	0.80	15.7	3.50 - 6.70
Serine + threonine + asparagine	7.37	2.20	29.9	2.97 - 11.77	3.30	11.80	4.88	0.76	15.6	3.36 - 6.39
Glutamic acid	0.67	0.29	0.43	0.20 - 1.25	0.20	1.36	1.12	0.22	19.6	0.72 - 1.60
Proline	9.12	1.65	18.1	5.82 - 12.42	5.76	12.54	5.86	1.18	20.1	3.49 - 8.22
Glycine	0.37	0.13	35.1	0.11 - 0.63	0.18	0.66	0.30	0.07	23.3	0.16 - 0.43
Alanine	1.49	0.62	41.6	0.25 - 2.73	0.74	2.76	1.53	0.26	17.0	1.01 - 2.10
Valine	0.19	0.08	42.1	traces - 0.32	traces	0.32	0.19	0.04	21.0	0.12 - 0.27
Isoleucine	0.09	0.09	100.0	traces - 0.32	traces	0.32	0.05	0.04	80.0	traces - 0.18
Leucine	0.16	0.08	50.0	traces - 0.34	traces	0.34	0.05	0.03	75.0	traces - 0.11
Tyrosine	0.05	0.04	80.0	traces - 0.16	traces	0.16	0.02	0.02	100.0	traces - 0.10
Phenylalanine	0.08	0.09	113.0	traces - 0.32	traces	0.32	0.08	0.05	62.5	traces - 0.16
γ-aminobutyric acid	3.33	1.02	30.6	1.58 - 5.54	2.08	5.78	3.19	0.76	23.8	1.68 - 4.71

[a] n = number of samples
[b] Standard deviation
[c] Coefficient of variation
[d] 95% confidence interval

concluded that increased mineral content can be an indication of pulpwash. Yet, these characteristics should be only a part of more complete detection scheme, due to the anticipated variability of minerals content in the water used in pulpwashing, and other factors which may play an active role – such as location, season, etc.

Chlorides, sulphates. Two additional attributes exhibiting a wide difference in concentration between juice and peel were chlorides and sulphates. The mean values found in the peel of both grapefruit and orange were roughly double than that of the corresponding juices (Tables 1 and 3).

Total pectin, total flavonoids. Total pectin is normally present in a higher concentration in the peel than in the juice. Royo-Iranzo et al. (1977) reported that the pectin content in orange peel extract was much higher than in orange juice. Results (Tables 1 and 3) showed concentration differences with an almost sevenfold ratio. The range for orange juice found (0.30–0.81 g/kg) is in close agreement with values reported for California Navel orange juice as reported by Park et al. (1983). They pointed out that a high pectin content may indicate the presence of pulpwash in orange juice. The broad range which seems to characterize pectin content reduced the significance of this measurement as a simple and singular tool for detecting adulteration. Nevertheless, it is an important characteristic that should be considered.

Total flavonoids was found to be approximately one order of magnitude higher in the peel than in juice, in both orange and grapefruit. Hence, the flavonoids may be an important factor in detecting the addition of peel extract to natural citrus juices or concentrates.

Chloramine-T number. Higher values were found in the peel (Tables 1 and 3). This finding is utilized in routine analytical parameters determined for establishing citrus juice adulteration (Cohen et al., 1983a, b; Lifshitz et al., 1974).

Formol number, free amino acids. The formol number is usually used in quality control or in detection of adulteration of citrus juices and concentrates (Cohen et al., 1983a, b; Lifshitz et al., 1974; Wallrauch, 1980, 1981a, b). The ratio of formol number/proline may be used also to detect gross dilution and adulteration with addition of amino acids. However, our data did not indicate significant differences between juice and peel.

The main difference in the amino acids content between citrus peel and citrus juice was noticeable in the arginine concentration, which was much lower in the peel than in juice (Tables 2 and 4). Similar findings were reported by Petrus and Vandercook (1980). Hence, it is strongly suggested that arginine should be considered in tests to detect adulteration of orange and grapefruit juices.

In conclusion, significant differences between natural juices and peel extracts were observed mainly in the following attributes: isocitric acid, the ratio of acidity/isocitric acid, ash, calcium, total pectin, total flavonoids, chloramine-T number and arginine. These findings may be utilized as guidelines for detecting addition of peel extract to natural citrus juice. However, almost all values studied had maximum ranges in juice which overlapped with minimum ranges in peel extracts or vice versa. Thus, there do not appear to be many distinct levels of specific parameters that could be used individually for adulteration detection. Hence, a profile of several attributes will have to be developed for this purpose. This projects the need for a wide data base of citrus juice analyses and further research in combining statistical methods and multiconstituents analyses for the determination of citrus juice purity.

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Recovery of Citrus Cloud from Aqueous Peel Extract by Microfiltration

ILAN SHOMER and UZI MERIN

ABSTRACT

Microfiltration of reconsituted aqueous peel extract resulted in linear flux decline with time. Feed stream of reconstituted peel extract does not foul the membrane and flux decline is due solely to increased retentate viscosity. Cloud was concentrated up to 6.3% of washed dry matter, compared with 1.5% achieved by vacuum heat concentration. Permeate was at constant low viscosity during filtration experiment, free of insolubles, and crystal clear. Soluble sugars do not contribute to fouling gel layer; their permeation flux is constant, and is only a function of viscosity. Soluble high-molecular-weight polymers, such as pectins, cause membrane fouling and flux depression. Fluxes for soluble constituents, sucrose and pectin, were higher than those of reconstituted peel extract with similar viscosity.

INTRODUCTION

EXTRACTED STABLE CLOUD which is derived from citrus fruit peel includes relatively very small but significant amounts of insoluble particles in an aqueous media. The soluble constituents, mainly sugars, comprise most of the solids and after a vacuum heat concentration process constitute the factor which is known to determine the cloud strength by the industry. This is true even when the concentrated suspension is produced as an additive which is designed to give the natural turbid appearance of beverages. The cloud is known to be marketed as a suspension which includes both the total concentrated solubles and insolubles. It is reasonable to assume that differential removal of solubles and water may significantly increase the concentration of the insolubles and their stabilizing soluble agents.

Two separation techniques which are based on differential concentration of components due to their size, are differential continuous centrifugation and membrane filtration.

Continuous centrifugation is well established in the citrus industry, for the removal of coarse particles from a stream of suspension with stable insoluble particles. Further separation of a clear serum from fractions of stable insolubles by centrifugation is not economically feasible.

Membrane filtration could be adopted in the citrus industry for differential separation of insolubles from solubles. Separation by membranes results in two main streams: (1) a concentrate of insoluble and some soluble constitutents, and (2) an aqueous permeate with soluble solids of defined molecular size.

Continuous membrane filtration was applied to various foodstuffs in the form of reverse osmosis (RO), ultrafiltration (UF) and microfiltration (MF), and the variations between the different processes are well defined (Cooper, 1980; Flinn, 1970; Mears, 1976). Subjecting milk and cheese whey to RO and UF is well documented (Fenton-May et al., 1971, 1972; Glover et al., 1978). Other food streams which were processed using membrane filtration techniques include, for example, UF of vegetable protein (Cheryan, 1980; Manak et al., 1980) and RO of mandarin and tomato juice (Watanabe et al., 1979; Merson et al., 1980). Matthews et al. (1981) mentioned an UF process for treating single-strength cloud liquid prior to evaporation, in order to reduce the content of limonin and sugars while retaining the cloud principles. Recently, MF was introduced for the treatment of cheese whey and brine, and successfully removed fat, particles and microorganisms from the treated streams (Tanny et al., 1982; Merin et al., 1983a, b). Differential membrane separation enables retention of aroma, taste and color substances. Consequent concentration of solubles in the permeate may be improved in the absence of insolubles and soluble polymers of high molecular weight.

The present work examined the application of membrane filtration to citrus fruit peel extract in order to increase the differential concentration of insoluble components and soluble solids of high molecular size.

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MATERIALS & METHODS

Aqueous peel extract

Reconstituted aqueous peel extract of citrus fruit was used for the filtration trials. The liquid was adjusted from 60°Brix to 5°Brix by dilution with deionized water.

Pectin solution

High methoxy pectin (HMP) was dissolved in distilled water or in the permeate of the reconstituted peel suspension after filtration. Pectinase (Ultrazyme-100) was added in order to degrade the pectin. This degradation was done in order to adjust the concentration of 1 and 2% of HMP to viscosity levels which were obtained by 0.5% of nondegraded HMP solution. High concentrations of sucrose solutions of 40 and 50°Brix were compared for their filtration performance, with a viscosity similar to that of 0.5% HMP solution. The viscosity was measured by an Oswald viscometer 30°C and results are expressed in centistokes (cs). Pectinase inactivation was done by heating to 90°C for 10 min and the untreated solution was incubated and heat-treated as above. The solutions were immediately cooled to 5°C before further processing.

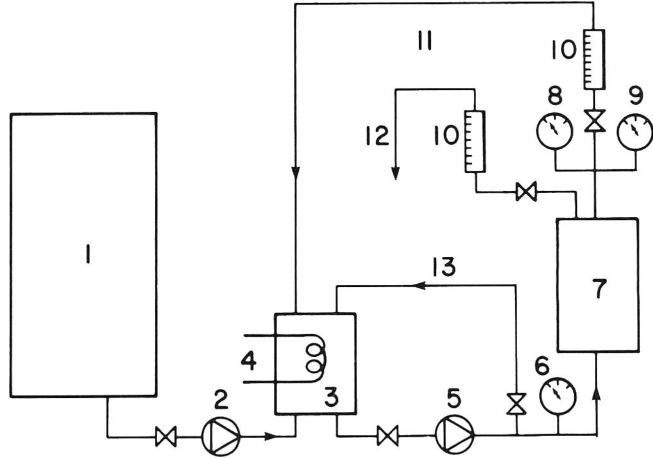

Fig. 1—A schematic diagram of the filtration apparatus: (1) Feed tank; (2) Feed pump; (3) Surge tank; (4) Cooling system; (5) Circulation pump; (6) Inlet pressure gauge; (7) Filtration module; (8) Outlet pressure gauge; (9) Temperature gauge; (10) Flow meters; (11) Retentate line; (12) Permeate line; (13) Bypass loop; (⋈) valves.

Filtration apparatus and procedure

A tangential flow Acroflux (TM) microfiltration device with a $<1\mu m$ pore size membrane was obtained from Gelman Sciences Inc. (GSI, Ann Arbor, MI). A schematic diagram of the filtration setup is shown in Fig. 1. The centrifugal pump was a 15 L/min 316 SS pump, operated at 85 kPa P_{AV} (135 kPa – P_{in}; 35 kPa – P_{out}), resulting in 12 L/min crossflow feed velocity, which is well in the turbulence flow region for the Acroflux capsule. Variables measured included: flux (L/m^2 hr), average pressure (P_{AV}, kPa), solution viscosity (centistokes), percent of washed dry matter and °Brix. Cloud concentration was expressed as percent washed dry matter.

Membrane cleaning procedure

Membrane cleaning was accomplished using an enzyme wash (0.005% Ultrazyme-100) followed by 0.1N NaOH. The cleaning cycle was as follows: H_2O rinse, 2 min; enzyme wash, 5 min; NaOH wash, 5 min; H_2O rinse, 5 min.

RESULTS & DISCUSSION

THE CONCENTRATION PROCESS of the reconstituted aqueous peel is shown in Fig. 2. There was a steady decrease in the permeation flux while there was a constant increase in the retentate viscosity; a phenomenon which is different from that reported for other MF feed streams (Merin et al., 1983a, b,). Unlike other membrane processes, where flux was reported to drop rapidly in the initial stages of the runs (Donnelly et al., 1974; Kuo and Cheryan, 1983; Lim et al., 1971; Merin and Cheryan, 1980), the flux depression in this case was due mainly to increased viscosity of the retentate. At the same time, permeate viscosity was very low and steady, and the permeate was crystal clear. Permeation flux decline was in direct proportion to the percent of washed dry matter (Fig. 2), which represents the cloud concentration. However, it should be noted that the permeate viscosity remained constant at 0.76 cs. This type of flux decline curve does not fit any of the pore-blocking mechanisms suggested by Grace (1956), or the flux decline equations suggested by Matthews et al. (1978) and by Merin and Cheryan (1980). This phenomenon points to the possible presence of particles with a consdierably larger size than the membrane pore size. The direct relationship of the percent dry matter and viscosity of the retentate to the average operating pressure (Fig. 3) suggests a nonfouling behavior of the feed stream in the 100-140 kPa P_{AV} operating pressure region practiced.

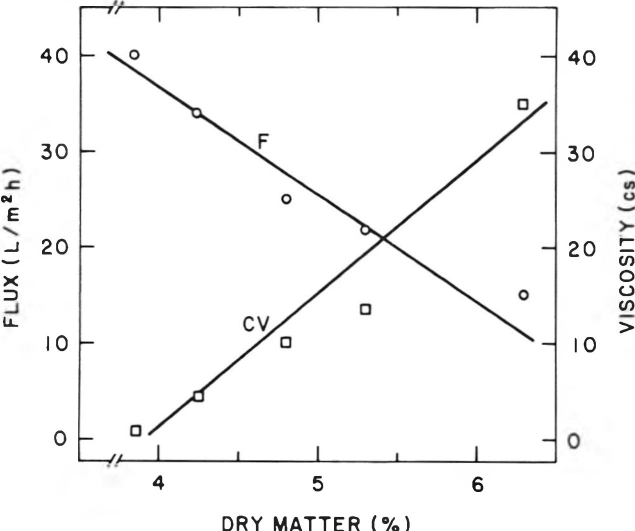

Fig. 2—Flux decline and concentrate viscosity of reconstituted aqueous peel extract with increase in dry matter, using a pilot-scale microfiltration module: F = Flux; CV = concentrate viscosity. F: $y = -9.95x + 75.78$, $r^2 = -0.98$; CV: $y = 13.48x - 53.46$, $r^2 = 0.97$.

It may be concluded, that an operating pressure above that which was tested (which was limited by the filtration apparatus) would have resulted in a higher permeate flux. This finding is of interest when compared with heat vacuum concentration. In the latter, only water is removed from the concentrating stream, and the final cloud concentration is limited by the solubles, mainly sugars, to 1.5% insoluble cloud constituents. In the case of membrane filtration, a greater than 400% increase in soluble cloud concentration was observed. It is obvious that the main contributor to the viscosity of the retentate was the insoluble components which are being retained by the membrane due to their molecular size. This assumption is based on the comparison of fluxes for an 8.0 cs peel extract retentate, which contained about 4.6% washed dry matter (Fig. 4A), compared with a 50°Brix sucrose solution of 8.1 cs and approximately 70% dry matter (Fig. 4).

So far, it can be stated that two major factors affected aqueous peel extract permeation rates: (1) the viscosity of the solution per se, which is attributed to the density and the molecular size of the soluble components; and (2) the dimensions and content of the insoluble particles as well as possible physical and chemical binding. In order to better understand the above mentioned phenomenon, two types of soluble feed streams were examined: (1) a sucrose solution of two concentrations, 40 and 50°Brix, with viscosity of 4.0 and 8.1 cs, respectively; and (2) high methoxy pectin (HMP) solution of various concentrations and molecular sizes – 0.5% nondegraded HMP, and 1 and 2% HMP solutions degraded by a pectinase, with viscosities of 4.2, 1.5 and 2.7 cs, respectively.

With a sucrose solution of 40 and 50°Brix, an increase in pressure resulted in increased flux (Fig. 4). This linear increase in flux with pressure suggests free permeation of solutes through the membrane, which was expected for the low molecular weight sucrose. Permeation fluxes were steady for a long period of time at the different average operating pressures used. When the data points of the same viscositites and pressure of the peel extract were compared with the sucrose performance, they corresponded to a

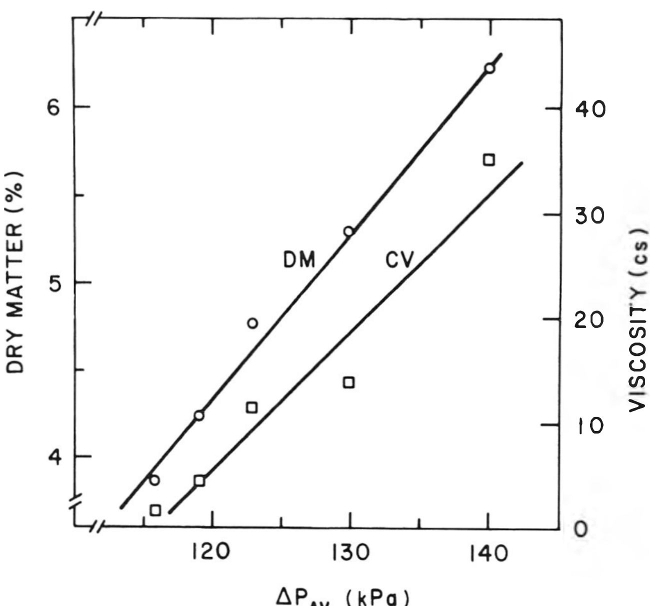

Fig. 3—Increase in dry matter and concentrate viscosity of reconstituted aqueous peel extract with an increase in average operating pressure, using a pilot-scale microfiltration module: DM = dry matter; CV = concentrate viscosity. DM: $y = 0.10x - 7.66$, $r^2 = 0.99$; CV: $y = 1.32x - 152.5$, $r^2 = 0.97$.

much lower flux (Fig. 4-A, B). Since the contribution of sucrose to the solution is limited to viscosity and it does not have the effect of a large molecular size component, HMP solutions were prepared as described earlier. Pectin, besides contributing to elevated viscosity, has a large molecule which is supposed to be larger than the membrane's pores size. High methoxy pectin, in which most of the charged carboxylic groups are methoxylated, seemed to behave as an unlinked polysaccharide. This soluble pectin was used as a model feed solution. The degraded polymer, obtained by the pectinase action, resulted in a smaller molecular size and thus in lowered viscosity. The viscosities of the three treatments and their permeation rates are presented in Fig. 5. The permeation fluxes of the 1 and 2% degraded HMP solutions were in close approximation to one another when corrected for their viscosities, and, although with a much higher HMP concentration, had fluxes similar to those of the nondegraded solution. It is suggested that the degree of degradation affected the permeation flux and obviously the retentate and permeate viscosities.

The nondegraded polymer was totally retained by the membrane, since its permeate viscosity was equal to that of pure water. The degraded polymers permeated the membrane to a certain degree, as is shown in their respective permeate viscosities. The examination of the contribution of HMP to the viscosity of the solution and to the consequent flux decline may be indicative of the role and function of the soluble polymeric components present in the juice suspension during the concentration process. It is postulated that nondegraded HMP has a pronounced effect on the filtration performance. When the polymer was broken down to small molecules, viscosity decreased and permeation flux increased. Nevertheless, if the degradation is limited (as is the case in the 2% HMP solution, Fig. 5), there may be some large enough molecules which will eventually block the pores (despite being soluble, as is the case with cheese whey proteins), and thus result in a decreased flux. When the degradation is more complete (Fig. 5 – 1% HMP solution), viscosity is much lower and consequently flux is much higher.

In order to understand the influence of pectin on the filtration performance, permeate-HMP solutions of permeate of peel extract and varying amounts of HPM were prepared. The contribution of the added HMP to the viscosity and its filtration performance is shown in Fig. 6. An increased viscosity with increased HMP concentration was expected, as was a decrease in permeation flux. Interesting is the flux decline behavior, which suggests the formation of a fouling layer which agrees with the phenomena discussed by Merin et al. (1983a, b) for the MF of cheese

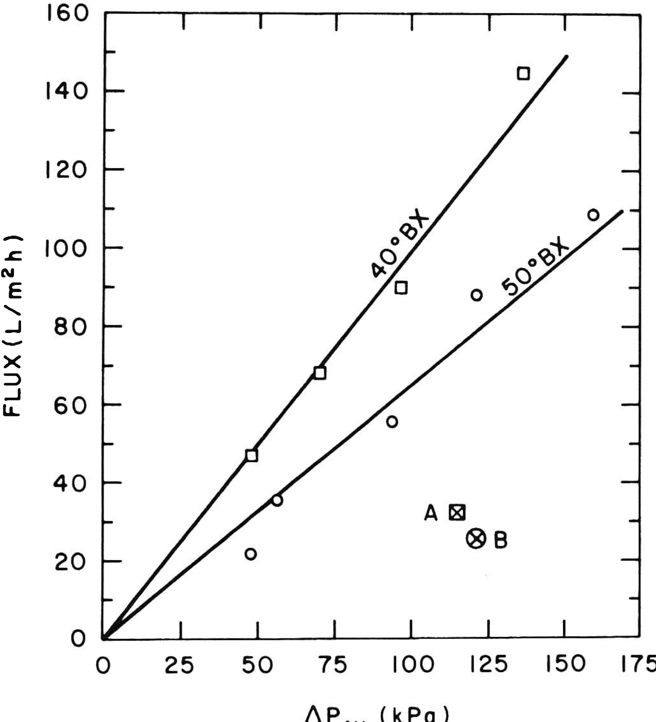

Fig. 4—Flux as a function of average operating pressure of 40 and 50°Brix sucrose solutions, using a pilot-scale microfiltration module: A = flux of a 4 cs aqueous peel extract; B = flux of an 8 cs aqueous peel extract.

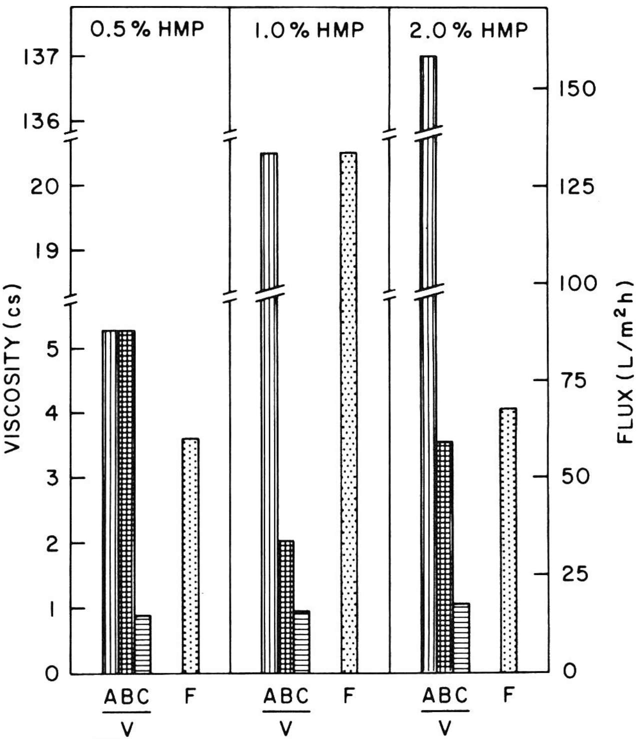

Fig. 5—A schematic diagram of viscosities and flux of different concentrations of HMP solutions: V = viscosity; A_V = before pectinase treatment; B_V = after pectinase treatment; C_V = permeate; F = flux.

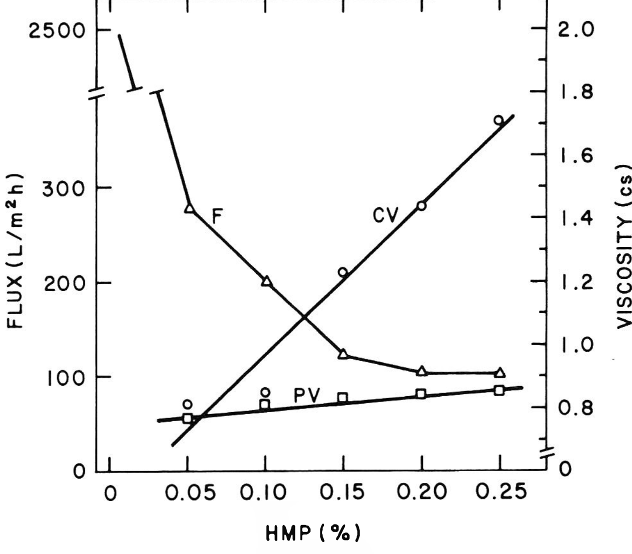

Fig. 6—Flux decline and concentrate and permeate viscosity with increased HMP concentration, using a pilot-scale microfiltration module: F = flux; CV = concentrate viscosity; PV = permeate viscosity. CV: $y = 0.000032x + 0.78$, $r^2 = 0.94$; PV: $y = 0.0048x + 0.49$, $r^2 = 0.98$.

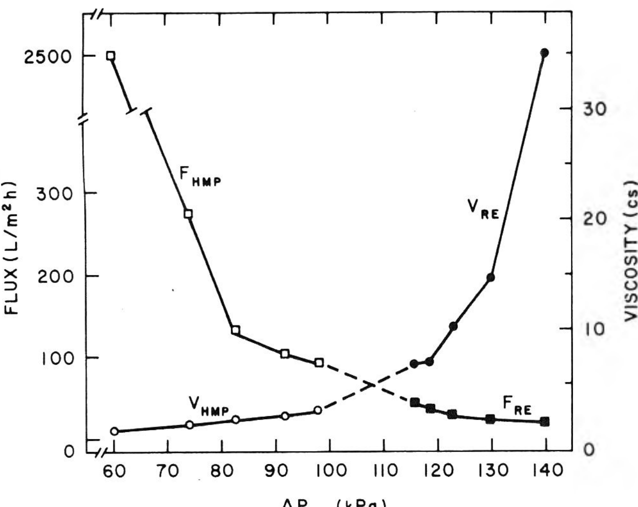

Fig. 7—Flux decline and increase in viscosity of permeate-HMP and reconstituted peel extract with an increase in average operating pressure, using a pilot-scale microfiltration module: F_{HMP} = flux of permeate-HMP (see text); F_{RE} = flux of reconstituted peel extract; V_{HMP} = viscosity of permeate-HMP retentate (see text); V_{RE} = viscosity of reconstituted peel extract retentate.

brine and cheese whey. Despite the increase in viscosity over 0.15% added HMP in the permeate-HMP solution (Fig. 6), there was almost no decrease in permeation flux after that point. It is assumed that the formed fouling gel layer achieved equilibrium. At this stage, the rate of deposition of a substance from the feed stream on the fouling layer is equal to the rate of back diffusion from the gel layer to the bulk (Cheryan, 1977; Kuo and Cheryan, 1983; Merin and Cheryan, 1980).

When the two systems (the reconstituted peel extract and the permeate-HMP solutions) were plotted on the same coordinates (Fig. 7), they complemented one another in a reciprocal manner. Flux declined very fast at very low viscosities (in the permeate-HMP solution), while viscosity increased sharply at low fluxes (compared with the permeate-HMP solution) of reconstituted peel extract. The dotted lines suggest the behavior of missing data points of various degrees of pectin concentration and their corresponding viscosities.

In conclusion, the microfiltration of reconstituted aqueous peel extract achieved a much higher concentration of insoluble peel extract constituents than could be obtained by heat vacuum concentration. Nevertheless, filtration of reconstituted peel extract is limited by the viscosity of the concentrated stream, which is due mainly to the insoluble components of the juice. The pectin in the juice, although being soluble, might also be a limiting factor during concentration, but its retention contributed significantly to the stability of the insolubles in the possible reconstituted beverage. Soluble sugars, on the other hand, do not seem to interfere with the performance of the microfilter.

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Application for Near Infrared Spectroscopy for Predicting the Sugar Content of Fruit Juices

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ABSTRACT

Near infrared spectroscopy, operated in the transmission mode, was used to predict the total sugar content of a variety of fruit juices. Attempts were made to determine individual sugars in some of the juices. The best results were obtained for total sugar determination when separate calibrations were used for each type of juice. For orange juice, when n=35, r=0.87, the standard error of calibration = 0.22, and the bias and standard error of prediction = 0.04 and 0.25 respectively. Minimal amount of sample preparation was required for the rapid and nondestructive analysis of the total sugar content of juices. The potential application and limitations of the method are discussed.

INTRODUCTION

ACCORDING TO Beverage World (Anon., 1977) the annual per capita consumption of fruit juices in the United States is 6.5 gallons; while total production is 1.4 billion gallons. The sugar content of juices is usually estimated with a hydrometer and reported as degrees Brix. This is really a measure of total solutes including sugar, and gives no data on the concentration of either individual sugars or total sugars. Near infrared spectroscopy (NIR) has proven itself to be a rapid, nondestructive, and accurate technique for a wide range of analytical applications (Osborne, 1981), and has emerged as a viable alternative to the slower more laborious wet-chemical methods (Cooper, 1983). NIR instrumentation requires calibration of the instrument by a primary laboratory method for each particular compound. Linear least squares regression analysis between NIR data and traditional chemical data yields a prediction equation which is then used to quantitate the same constituent in unknown samples. This makes NIR an ideal choice for quality control monitoring and for the analysis of a large number of similar samples. NIR has been used for determining total reducing sugars in tobacco (McClure et al., 1977), and individual sugars in dry fruit model systems (Giangiacomo et al., 1981).

Conventional NIR reflectance measurements are unsuitable for fruit juice samples because their high water content allows most of the NIR light to pass through the sample without being reflected. We, therefore, investigated the possibility of using NIR transmission measurements to quantitate the sugar content of juices. The total sugar content and the individual sugars; glucose, fructose, and sucrose of various types of ready-to-serve juices were examined in this study.

MATERIALS & METHODS

Instrumentation

All near infrared (NIR) measurements were made with a Pacific Scientific Model 6350 Spectrocomputer (Pacific Scientific, Silver Spring, MD). This instrument has a single-beam scanning monochromator that provides a linear scan over the 1100–2500 nm region. The Model 6350 comes equipped with both reflectance and transmission capability, in the transmission mode one lead sulfide cell is positioned directly beneath the sample. An empty quartz transmission cell was used as the reference standard. The absorbance data for both the sample and standard were recorded as log 1/T (T=transmittance).

NIR procedure

Four distinct steps are required for using NIR for quantitative analysis. First, NIR as currently used is a semiempirical technique and requires calibration using data from a primary or a secondary method (Cooper, 1983). Therefore, a set of samples, a calibration set, is analyzed by a recognized laboratory method. Fruit juice samples were analyzed by gas-liquid chromatography (GLC) for quantitation of individual sugars, and a flow injection analysis system was used for total sugar determination. The NIR spectra for each calibration set is then correlated with the chemical laboratory data. A step-foward linear least square regression yields an equation of the form:

$$Y = b_0 + \sum_{i=1}^{n} K_i \ \alpha_i$$

where Y is the concentration of a constituent in the product, n is the number of terms in the equation, b is a constant, K is the regression coefficient, and α is a mathematical transformation of the raw optical data measured at any wavelength. Instead of correlating the chemical data to the log(1/T) data were correlated to the second derivative transformation of the log(l/t) data, since derivative transformation of spectral data not only sharpens the details in the spectra, but also reduces baseline shifts (Norris and Barnes, 1976). This chooses the wavelengths at which the absorption data best correlates to the concentration, and calculates the regression coefficients. The specific form of the multiple regression equation used in our study to select from one to four wavelengths is:

$$Y = b_o + K_1 \frac{\dfrac{d^2\log(1/T_{\lambda_1})}{(d\lambda_1)^2}}{\dfrac{d^2\log(1/T_{\lambda_2})}{(d\lambda_2)^2}} + K_2 \frac{\dfrac{d^2\log(1/T_{\lambda_3})}{(d\lambda_3)^2}}{\dfrac{d^2\log(1/T_{\lambda_4})}{(d\lambda_4)^2}}$$

A different calibration equation is needed for each constituent being quantitated. The calibration is evaluated in terms of accuracy and precision using another set of samples, usually called the prediction set. These samples, after being analyzed by the same chemical technique as the calibration set, are run on the NIR instrument and the sugar concentration predicted using the recently derived calibration equation. A comparison of the bias and standard error between the chemical and NIR data is then performed. A more detailed description of the instrument, the instrument calibration, and the mathematical transformation of the spectral data were published previously (Lanza, 1983).

Chemical analysis

The total sugar content (the sum of glucose, fructose, and sucrose) of fruit juices was determined by an automated, flow-injection technique for the colorimetric analysis of sugars. This total sugar analyzer (TSA), which was developed in our Laboratory, initially hydrolyzes sucrose to glucose and fructose in 1.0N HCl. The reaction of glucose and fructose with p-hydroxybenzoic acid hydrazide is then quantitated colorimetrically. For a selected number of juices the individual sugars, glucose, sucrose, and fructose were

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determined by a gas-liquid chromatographic (GLC) method using trimethylsilylated oxime derivatives. Li and Schuhmann (1983) described both of these procedures in more detail.

Fruit juice samples

Eleven different types of fruit juices – apple, citrus blend, cranberry, grape, grapefruit, lemonade, orange, pineapple, prune, tomato, and vegetable – were analyzed. Our calibration for total sugar for all juices were derived using 6 samples from each of these 11 types of juices. The prediction set for all juices consisted of 3 samples of each type except for orange juice which had 4 samples. The calibration for individual sugars for all juices consisted of 33 samples, 3 from each of the eleven types. The only sample preparation required for juices before NIR analysis was centrifugation at 1000 rpm for 10 min to remove pulp. Three milliliters of the resultant supernatant were pipetted into the NIR sample cell (pathlength = 2.2 mm) for NIR scanning.

Standard sugar solutions

Twenty aqueous solutions each containing different amounts of glucose, fructose, and sucrose were made up to resemble the sugar content of the eleven types of juices analyzed in this study.

Statistics

The standard error of calibration (SEC) measures how well the instrument matches calibration samples and is calibrated by

$$SEC = \left(\frac{\sum_{i=1}^{n} (Y_i - \hat{Y}_i)^2}{n-3} \right)^{1/2}$$

The bias is calculated as

$$BIAS = \frac{1}{n} \sum_{i=1}^{n} (Y_i - \hat{Y}_i)$$

Table 1—Wavelengths selected and statistical summary of glucose, sucrose and fructose solutions determined by NIR analysis[a]

	λ_1[b] (nm)	λ_2 (nm)	$\bar{X}$[c] (%)	r	SEC[d] (%)
Glucose	1402		15.0	1.00	0.08
Fructose	2274		15.0	1.00	0.02
Sucrose	2268	2254	15.0	1.00	0.07

[a] For each NIR calibration 20 aqueous solutions of a particular sugar containing 1-20% sugar were prepared.
[b] λ_1, λ_2 refer to the wavelengths selected for the calibration equation described in Materials & Methods.
[c] Mean value for the calibration samples.
[d] Standard error of the calibration samples.

The standard error of prediction (SEP) is the standard deviation of the prediction error

$$SEP = \left(\frac{\sum_{i=1}^{n} [(Y_i - \hat{Y}_i) - Bias]^2}{n-1} \right)^{1/2}$$

where n = number of samples, Y = laboratory concentration, and $\hat{Y}$ = NIR predicted concentration.

RESULTS & DISCUSSION

IN ORDER TO DETERMINE the applicability of NIR for sugar analysis in fruit juices, we first examined aqueous solutions containing only one sugar, either glucose, fructose, or sucrose. Even though the transmission spectra of 50% aqueous solutions of each of these sugars were not very different from the spectra of water, except for small differences in the 2270 nm region (Fig. 1), these solutions still had a correlation coefficient of 1.00 and a low standard error of calibration (SEC) (Table 1). However, solutions containing various combinations of all three sugars had SEC values at least 5 times higher than when only a single sugar was present (Table 2). Since the overlap in the NIR spectra of these sugars in the 2270 nm region (Fig. 1) could not even be resolved using second derivative transformation of the spectra (Fig. 2), it appears that the increased SEC shown in Table 2 was due to interferences between the sugars. Previous studies quantitating sugars by NIR have been done in dry materials, such as tobacco or freeze-dried fruits. In these substances sugars are in their crystalline form and have considerably more spectral detail, as illustrated in Fig. 3. The above figures illustrate the difficulties in using NIR for quantitative analysis of high moisture samples. The well known water absorptions around 1450 nm and 1900 nm are so intense that they obscure other spectral detail, and, in addition, since the sugar absorbances in the NIR region are more sugar-water than sugar-sugar interactions observed in crystalline structures, all sugars appear quite similar.

The quantitation of total sugar by NIR was done using either one calibration for all 11 different types of juices, or using separate calibrations for orange juice, apple juice, and lemonade (Table 3). When a single calibration was used for all 11 types of juices the standard error of prediction (SEP) was 50% larger than the SEC. Most of this error was produced by two types of juices, pineapple and grape. Pineapple juice even after centrifugation had more pulp than other samples, which could lead to an increased amount of

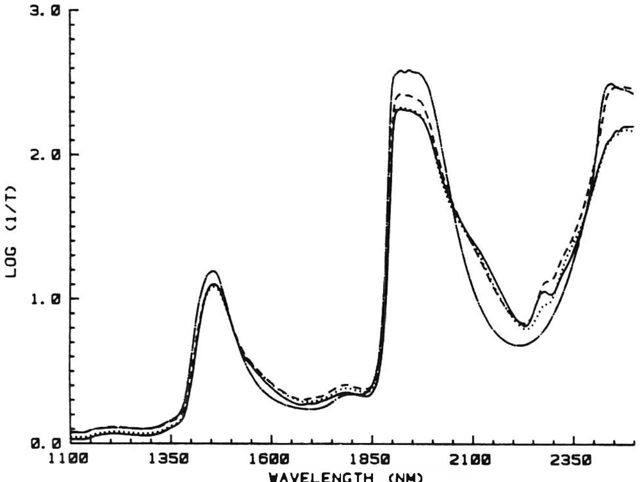

Fig. 1—NIR spectra of 50% solutions of glucose, fructose, sucrose and pure water: Glucose ——; Sucrose – – – –; Fructose · · · · ·; Water -·-·-·-·.

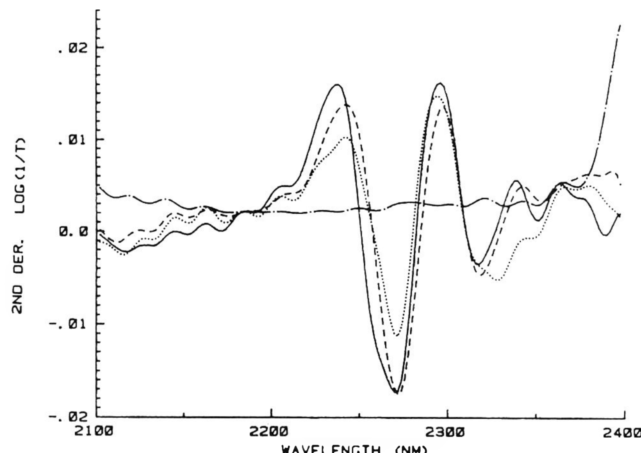

Fig. 2—Second derivative plots of the NIR spectra of 50% solutions of glucose, fructose, and sucrose: Glucose ——; Sucrose – – – –; Fructose · · · · ·; Water -·-·-·-·.

light scattering, and grape juice had a much higher proportion of fructose than the other juices. The best results were obtained for orange juice, in which the SEC was nearly the same as the SEP and the bias was relatively small indicating the correctness of the calibration equation. For apple juice and lemonade the SEC and the SEP were nearly the same, however, the bias was higher probably due to the limited sample size for these two juices. The SEP for orange juice was 0.25%, indicating a 0.25% difference between NIR procedure and the TSA procedure for determining total sugar content. In order to decide if the magnitude of this error was acceptable we compared the difference between the TSA procedure and the analysis of total sugar by GLC (the summation of glucose, fructose, and sucrose), using a paired-t test (Snedecor and Cochran, 1967). The difference in this case was only 0.12%. Since for random errors, Sy = Sy/n, the SEP for NIR could be reduced to the lower value by analyzing additional samples. This could be easily done since NIR analysis time was less than a minute per sample.

At least one of the wavelengths selected by the regression equation should correspond to an area of significant absorption by the constituent being measured compared to that of any other terms in the prediction equation. This avoids indirect measurement of a constituent and helps guard against possible overfitting of the calibration equation (Cooper, 1983). Since the first term of the regression usually carries most of the weight of the multiple regression equation, λ_1 should correspond to a sugar peak. The role of λ_2 is not yet fully understood, but Norris (1983) has conjectured that the denominator measures the total amount of material rather than a specific constituent. For both orange juice and lemonade, sucrose was the predominate sugar and the wavelengths chosen were quite similar, but for apple juice, with 50% of its sugar content as fructose, the wavelengths were quite different (Table 3). The wavelengths found for orange juice and apple juice were 2268 nm and 2270 nm respectively, similar to the λ_1 found for pure aqueous sucrose (Table 1), but quite different from the wavelengths for sucrose in sugar mixtures, which indicated that the computer was selecting different wavelength regions to compensate for interferences from other sugars.

Analysis of individual sugars across all juices and for orange juice is given in Table 4. The low r values indicate that individual sugars could not be quantitated using these calibration equations, since in regression analysis when r = 0.7 only about one-half of the variance of the independent variable 'y' was associated with the calibration line. The low correlations were probably due to the overlap in the NIR spectra of individual sugars (Fig. 2). Although the SEP found for individual sugars in orange juice were comparable to the SEP for total sugar, they were merely a reflection of the narrow range of individual sugars in these samples. In fact, the standard errors for GLC replicate analyses for fructose, glucose, and sucrose were found to be +0.03%, +0.02%, and +0.05%, respectively.

CONCLUSION

FOR RAPID and nondestructive determination of the total sugar content, NIR spectroscopy, when operated in the

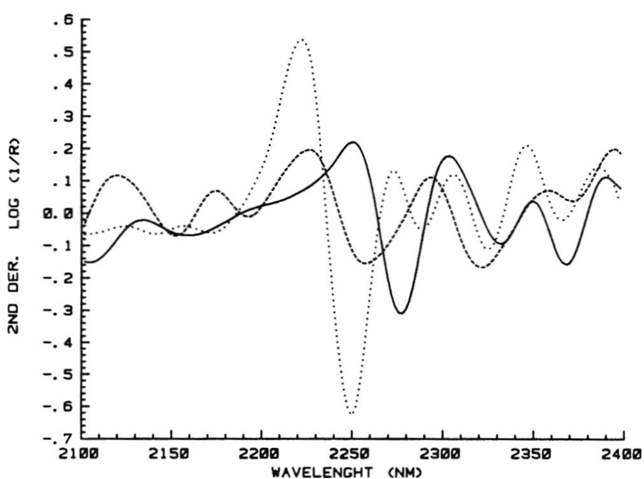

Fig. 3—Second derivative plots of the NIR spectra of powders of glucose, fructose, and sucrose: Glucose ——; Sucrose — — —; Fructose · · · · · · ; Water · · · · · · · ·.

Table 2—Wavelengths selected and statistical summary of aqueous solutions[a] containing mixtures of glucose, sucrose and fructose determined by NIR spectroscopy

	λ_1[b] (nm)	λ_2 (nm)	λ_3 (nm)	λ_4 (nm)	$\bar{X}$[c] (%)	r	SEC[d] (%)
Glucose	2290	2198	1800	2190	2.67	0.98	0.41
Fructose	2256	2314	1898	1260	3.40	0.99	0.21
Sucrose	1772	1704	2282	1348	2.33	0.98	0.38
TOTAL	2240	2186	1402	2236	8.40	0.99	0.25

[a] Twenty aqueous solutions containing various concentrations of all 3 sugars as described in Materials and Methods.
[b] λ_1, λ_2, λ_3 and λ_4 refer to the wavelengths selected for the calibration equation described in Materials & Methods.
[c] Mean value for the calibration samples.
[d] Standard error of calibration.

Table 3—Wavelengths selected and statistical summary of total reducing sugar content of fruit juices by NIR spectroscopy

	λ_1[a]	λ_2	λ_3	λ_4	n_1[b]	$\bar{X}$[c] (%)	SEC[d]	r	n_2[b]	Bias	SEP[e]	Min – Max[f] (%)
All juices	2258	2240	1238	2268	66	10.02	0.48	0.99	34	0.14	0.73	2.22–14.90
Orange	2268	1206	–	–	35	10.40	0.22	0.87	10	0.04	0.25	9.31–11.30
Apple	1236	2272	2260	1964	9	11.30	0.42	0.97	8	0.11	0.46	9.12–13.50
Lemonade	2270	1212	–	–	13	11.20	0.32	0.98	10	0.14	0.39	9.66–13.04

[a] λ_1, λ_2, λ_3, λ_4 refer to the wavelengths selected for the calibration equation described in Materials & Methods.
[b] n_1 equals the number of calibration samples, n_2 equals the number of prediction samples.
[c] Mean value for the calibration samples.
[d] Standard error of the calibration samples.
[e] Standard error of the prediction samples.
[f] Min and Max % sugar for both the calibration and prediction samples.

Table 4—Wavelengths selected and statistical summary of individual sugar analysis in fruit juices by NIR spectroscopy

Sugar	Juice	λ_1[a]	λ_2	λ_3	λ_4	n_1[b]	$\bar{X}$[c] (%)	r	SEC[d]	n_2[b]	Bias	SEP[e]	Min–Max (%)
Glucose	All types	2290	1394	1496	2312	3.02	3.02	0.66	0.60	8	0.32	0.49	1.2–7.9
Fructose	All types	2254	1810	2310	1644	3.32	3.32	0.68	0.60	8	0.23	0.61	1.5–6.7
Sucrose	All types	2284	1680	1802	1486	3.23	3.23	0.51	0.43	8	−0.43	0.58	0 –5.6
Glucose	Orange	2270	1400			23	2.22	0.72	0.13	6	0.09	0.20	2.1–2.5
Fructose	Orange	2274	1400			23	3.03	0.68	0.24	6	0.20	0.26	2.8–3.3
Sucrose	Orange	2264	1748			23	5.25	0.71	0.35	6	0.09	0.35	5.0–5.6

[a] λ_1, λ_2, λ_3, λ_4 refer to the wavelengths selected for the calibration equation described in Materials & Methods.
[b] n_1 equals the number of calibration samples, n_2 equals the number of prediction samples.
[c] Mean value for the calibration samples.
[d] Standard error of the calibration samples.
[e] Standard error of the prediction samples.
[f] Min and Max % sugar for both the calibration and prediction samples.

transmission mode has potential application for the analysis of fruit juices or aqueous samples and could be used for routine quality control of food products. Individual sugars, however, cannot be determined with acceptable accuracy and precision under the conditions we have investigated.

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by initial riboflavin content or the partial photodegradation of riboflavin before cooking.

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Malic Acid Degradation and Brined Cucumber Bloating

R. F. McFEETERS, H. P. FLEMING, and M. A. DAESCHEL

ABSTRACT

Bloater formation of brined cucumbers increased as more malic acid was degraded to CO_2 and lactic acid. CO_2 production by the brined cucumber, unrelated to malic acid degradation, was 12.5 mM. This was just sufficient to bring cucumbers to the point of bloating. CO_2 from malic acid provided the marginal increase required to cause significant bloating. Fermentation with a strain of *Lactobacillus plantarum*, which did not degrade malic acid, prevented cucumber bloating. Oxygen exchange of cucumbers before brining increased the amount of CO_2 required to initiate bloating damage by 8 mM. Nonmalic acid-degrading starter cultures and/or oxygen exchange may be useful alternatives to CO_2 purging from brines to prevent bloater damage.

INTRODUCTION

MALIC ACID degradation to lactic acid and CO_2 is the major reaction which results in CO_2 production in cucumber juice by lactic acid bacteria (McFeeters et al., 1982). However, when cucumber fruit are brined, a substantial amount of CO_2 is produced by the fruit in the absence of microbial fermentation (Fleming et al., 1973). Multiple sources of CO_2 evolution in cucumber fermentations may elevate CO_2 concentrations enough to cause bloater defects (hollow centers). The relative contribution of CO_2 from different sources is important in devising ways to minimize CO_2 production in fermentations. Since CO_2 production from malic acid is caused by the fermentation bacteria, it is of interest to find organisms which do not degrade malic acid to determine whether bloating could be prevented by eliminating that source of gas production.

The objectives of this study were: (1) to determine the relationship between malate decarboxylation and bloater formation during fermentation of brined cucumbers, and (2) to compare the effects on bloater damage of lactic acid bacteria that decarboxylate malic acid with those that do not. For this purpose, 19 strains of *Lactobacillus plantarum* and 5 strains of *Pediococcus cerevisiae* were surveyed in an attempt to obtain nonmalic acid-degrading lactic cultures.

MATERIALS & METHODS

FOURTEEN STRAINS OF BACTERIA, designated as *Lactobacillus plantarum* with culture numbers 82, 340, 341, 343, 352, 354, 363, 963, 965, 1193, 1194, 1752, 1939, and 1988 were obtained from the National Institute for Research in Dairying (Reading, England). *Lactobacillus plantarum* YIT-0068 was obtained from Yakult Institute for Microbiological Research (Tokyo, Japan). *Pediococcus cerevisiae* 20 was provided by Dr. J.B. Evans, Dept. of Microbiology, North Carolina State Univ. (Raleigh, NC). *Pediococcus cerevisiae* 23 was from the late Dr. J.O. Mundt, Dept. of Microbiology, Univ. of Tennessee (Knoxville, TN). *Pediococcus cerevisiae* ABC was from A.B.C. Research Corporation (Gainesville, FL). *Lactobacillus plantarum* WSO, 15, 16, 442, and *P. cerevisiae* 61 were from the culture collection of this laboratory. *Lactobacillus cellobiosus* ATCC 11739, a heterofermentive species, was obtained from the USDA-ARS Northern Regional Research Center culture collection.

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A nutritive medium was designed to test for malic acid degradation in the bacteria listed above. The main factors considered for selection of an appropriate medium were that lactic acid bacteria would grow, the medium did not contain major sources of CO_2 other than malic acid, and it did not contain components that would interfere with HPLC determination of malic acid and lactic acid. One liter of medium contained 10g Difco peptone, 5g yeast extract, 2g KH_2PO_4, 0.2g $MgSO_4 \cdot 7\ H_2O$, 0.05g $MnSO_4 \cdot H_2O$, 10g glucose, and 6.6g L-malic acid. Each organism was grown in MRS medium (De Man et al., 1960) and then inoculated into the test medium. They were incubated at 30°C for 6 days, then fermentation products were analyzed.

Cucumber juice was prepared as described previously (McFeeters et al., 1982). NaCl (5% w/v) was added to the nondiluted juice. The juice was sterile-filtered into a Vacutainer tube through a Millipore 0.22 μm Millex filter. *Lactobacillus plantarum* strains WSO, 963, and 965 were grown in MRS medium with 2% NaCl (w/v), washed with sterile saline, resuspended in 4°C cucumber juice, and inoculated into triplicate tubes. After 7 days' incubation at 30°C, fermentation products were analyzed.

Pickling cucumbers (3.8–5.1 cm diameter) from commercial sources were fermented in 1-gal (3.78 liter) jars with a tightly closed lid to prevent CO_2 loss during fermentations. Rubber septa were put into the lids to make additions or take samples from jars without opening the lid. Jars were filled with an equal weight of cucumbers and a brine which contained 10% NaCl and 0.32% acetic acid. If malic acid were added, 20, 40 or 60 mL of a 17.6% solution of L-malic acid was added to the brine solution to give equilibrated malic acid concentrations 7, 14, and 21 mM higher than the concentration originating from the cucumbers. The cucumbers were held in this brine for 3 days to allow the salt concentration in the liquid to fall below 6%. The low pH and high salt concentration prevented initiation of a natural fermentation during this time. To raise the pH before inoculation of culture, 24g of sodium acetate trihydrate were dissolved in 30 mL of water. This was injected into the jars through the septum. If malic acid had been added to the cover brine, sufficient 50% NaOH solution was added to neutralize the malic acid. Half of the NaOH was added at the same time as the acetate. The remainder was added 2 days later. Once the pH had been raised to approximately 4.5, each jar was inoculated with 2 mL of a 16-hr culture of the appropriate organism grown in MRS medium with 2% NaCl. The cucumbers were held at 27°C for 3 wk and then analyzed.

Cucumbers were oxygen-exchanged for 30 min at a rate of 300 mL/min according to the procedure of Fleming and Pharr (1980). After brine was added to the exchanged fruit, the expansion tower was removed and replaced with a lid with a single rubber septum, as described above.

Before the closed jars of fermented cucumbers were opened, triplicate 10-mL brine samples were removed through the septum for CO_2 analysis. The cucumbers could not bloat in the closed jars because there was no space available for expansion. When lids were removed from the jars, a pressure differential developed, and the cucumbers bloated. Three hours after opening the jars, the fruit were examined for bloater damage. A sample for fermentation product analysis was prepared by blending an equal weight of brine and cucumber tissue. A half cross-sectional piece was cut from 10–12 cucumbers in each jar to obtain a representative tissue sample.

CO_2 analysis was done with the method of Fleming et al. (1974). The percentage of bloater damage was expressed as a bloater index value calculated according to Fleming et al. (1977). Concentrations of reducing sugars were determined with the dinitrosalicylic acid reagent (Sumner and Sisler, 1944). Malic acid, lactic acid, and acetic acid were analyzed by HPLC with an 8 × 100 mm, 10 μm C_{18}, reversed-phase Radial-Pak column (Waters Associates, Milford, CT) with pH 2.5, 0.05M phosphoric acid as the eluant (McFeeters et al., 1984).

RESULTS & DISCUSSION

OF THE 19 STRAINS of *L. plantarum* and 5 strains of *P. cerevisiae* that were evaluated for their ability to degrade malic acid in the nutritive medium, only *L. plantarum* 963 and 965 were found to lack the ability to completely degrade malic acid.

These presumptive, nonmalic acid-degrading strains were then compared with *L. plantarum* WSO for their ability to degrade malic acid and ferment cucumber juice containing 5% NaCl. The WSO strain, as has been previously shown (McFeeters et al., 1982), degraded all of the malic acid in cucumber juice (Table 1). However, less than 3% of the initial malic acid disappeared during a 7-day incubation period with strain 965. Strain 963 showed intermediate characteristics in that it degraded 22% of the malic acid. CO_2 production by WSO was equivalent on a molar basis to the malic acid metabolized, Both the 965 and 963 strains produced less CO_2 than WSO, but more CO_2 than could be accounted for by malic acid degradation. The difference between the total lactic acid formed during fermentation and the malic acid degraded by each strain was considered to be the lactic acid production from sugar metabolism. The WSO strain produced nearly twice as much lactic acid as the other two strains.

The three strains of *L. plantarum* shown in Table 1, along with a heterofermentative organism, *L. cellobiosus*, were used to ferment cucumbers to observe the relationship between CO_2 formation and bloating. Analysis of duplicate jars for malic acid and sugar degradation and lactic acid production is shown in Table 2. Cucumbers did not ferment initially when they were covered with salt and acetic acid. When the pH was raised and no inoculum was added, a natural fermentation occurred. However, when lactic acid bacteria were inoculated immediately after pH adjustment, the inoculated organism apparently dominated the fermentation. This was indicated by the fact that malic acid remained at the end of fermentation, whereas, malic acid is not found in natural fermentations because the natural lactic acid bacteria degrade malic acid. The fact that nearly 80% of the malic acid was degraded in fermentation A with the 963 culture indicated that some competition by natural malic acid-degrading organisms may have occurred.

Even though the 963 and 965 strains were isolated from cheddar cheese (Sherwood, 1939; strains 1.1 and 4.3, respectively), they did carry out an active fermentation of cucumbers under the conditions described. This is indicated by the fact that 85% of the sugars present in the cucumbers were fermented by 965 and 90-92% of the sugars by strain 963. This compares to complete sugar removal by the WSO strain, which is commonly used for cucumber fermentations. The fact that the 965 and 963 strains produced more acid in cucumbers than in juice fermentations was attributed to the fact that the acetate added buffered the cucumber fermentation.

The relationship between bloater index and brine CO_2 concentration when cucumbers were fermented with different organisms was linear (Fig. 1). The critical point below which bloating did not occur (Fleming et al., 1978; Fleming, 1979) was 12.6 mM CO_2. The 965-fermented cucumbers reduced the CO_2 production relative to the control WSO strain to the point that bloating was eliminated by use of the nonmalic acid degrading lactic culture. The CO_2 production in the jars 3 days after brining, before inoculation with 965, was 8.2 mM. Therefore, a mean of 4.4 mM CO_2 was produced during the fermentation period, compared to a mean of 10.3 mM CO_2 production for the WSO fermentations.

An experiment was then conducted to determine the relationship between malic acid degradation and CO_2 production in fermented cucumbers. Cucumbers with the natural level of malic acid were fermented with both *L. plantarum* 965 and WSO strains. Cucumbers supplemented with 7, 14, and 21 mM malic acid were fermented only with the WSO strain. A linear relationship between CO_2 production and malic acid degradation was observed (Fig.

Table 1—Malic acid, CO_2, and lactic acid in cucumber juice + 5% NaCl after fermentation with strains of L. plantarum for 7 days at 30°C

Strain	Malic acid (mM)	CO_2 (mM)	Lactic acid: Total (mM)	Lactic acid: From malic acid[a] (mM)	Lactic acid: From sugar[a] (mM)
Noninoculated	14.7 ± 1.0	0.3 ± 0.1	–[b]	–[b]	–[b]
WSO	–[b]	15.1 ± 0.3	134.1 ± 0.4	14.7	119.4
965	14.3 ± 0.6	2.5 ± 0.3	62.9 ± 1.6	0.4	62.5
963	11.5 ± 1.2	6.3 ± 1.5	64.4 ± 3.8	3.2	61.2

[a] Calculated on the assumption that one mole of lactic acid was formed for each mole of malic acid degraded and that the remainder of the lactic acid was produced by sugar fermentation.
[b] Nondetectable.

Table 2—Malic acid degradation, sugar degradation, and lactic acid production during fermentation of cucumbers with different lactic acid bacteria[a]

Organism	Fermentation[b]	Malic acid degradation (%)	Sugar degradation (%)	Lactic acid production (mM)
L. plantarum WSO	A	100	100	140.7
	B	100	100	128.4
L. plantarum 965	A	7	85	100.4
	B	26	85	99.1
L. plantarum 963	A	79	93	122.7
	B	19	90	102.6
L. cellobiosus	A	93	100	91.5
	B	93	100	51.2
Natural fermentation[c]	A	100	78	85.7
	B	100	97	63.8

[a] Calculated on a brined, equilibrated basis, the fresh cucumbers contained 10.0 mM malic acid and 85.8 mM reducing sugar.
[b] A and B indicate duplicate fermentation jars.
[c] Brine additions and adjustments were the same as the other four treatments, but no starter culture was added.

2). However, only 0.8 moles of CO_2 was produced per mole of malic acid degraded. This suggested that a small amount of the malic acid may have been utilized by either cucumber enzymes or the fermentation microorganisms by a reaction other than the malolactic reaction. In cucumber juice fermentations, a 1:1 ratio was observed (McFeeters et al., 1982). The juice used for those experiments had been heated to inactivate cucumber enzymes. The intercept of the curve in Fig. 2 provides an estimate of CO_2 produced in the fermentation from sources other than malic acid.

Fig. 3 shows the relationship between CO_2 formation and bloating in oxygen-exchanged and nonexchanged cucumbers. The relationship between bloater index and CO_2 production in the nonexchanged fruit was not linear, as occurred in the experiment shown in Fig. 1, even though the range of CO_2 production was similar. This probably represents differences in the physical strength of the cucumber carpels and the way in which carpel separation occurs in different lots of cucumbers. Further work is needed to characterize quantitative relationships between physical characteristics of cucumbers and bloating in response to CO_2. The cucumbers fermented with strain 965 showed slight bloating. Extrapolation to zero bloater index in the nonexchanged condition indicated that the critical CO_2 concentration was 12.4 mM. This bloating threshold was very close to that found in the experiment shown in Fig. 1. In both experiments, the CO_2 production attributable to the cucumber was sufficient to bring the cucumbers to the point at which bloating could begin. CO_2 production from malic acid provided the margin which caused significant bloating of the fruit. Prevention of malic acid degradation would, therefore, prevent most bloating in a controlled, homolactic acid fermentation of cucumbers.

Fleming and Pharr (1980) have shown that oxygen exchange of cucumbers before brining, to replace the air normally present in the gas spaces of the fruit with oxygen, reduces the susceptibility of cucumbers to bloating during fermentation. The results in Fig. 3 show an 8 mM increase in the concentration of CO_2 required to initiate bloating (20.8 mM vs 12.4 mM for nonexchanged fruit). This differential was maintained as the CO_2 concentration was increased by degradation of larger amounts of malic acid until extensive bloating had occurred. Thus, O_2 exchange could provide a margin of protection against bloating, even if some malic acid were degraded. The combination of fermentation with nonmalic acid-degrading bacteria and O_2 exchange in cucumber brining tanks could provide a considerable margin for prevention of bloating without the need to remove CO_2 by purging. A problem which remains to be solved before O_2 exchange can be applied under commercial conditions relates to undesirable bacteria being drawn into the cucumbers (Daeschel, 1982). Daeschel and Fleming (1981) have shown that bacteria enter the fruit through stomatal openings due to the partial vacuum which develops when O_2-exchanged fruit are put into brine (Corey et al., 1983).

Though the results with *L. plantarum* 965 demonstrate the desirability of using nonmalolactic bacteria in cucumber fermentations, it is not suitable as a fermentation organism with current procedures because sugars are not completely fermented. Results of our survey of 24 homofermentative lactic acid bacteria and the results of Caspritz and Radler

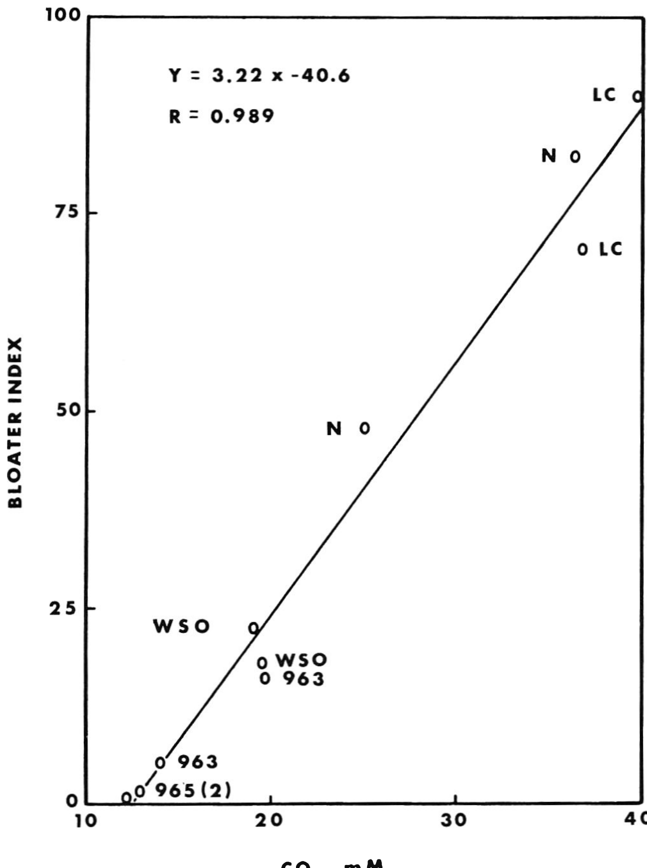

Fig. 1–Relationship between CO_2 production and bloater damage in cucumbers fermented with different microorganisms. The organisms used for each fermentation are indicated as follows: L. plantarum WSO, WSO; L. plantarum 963, 963; L. plantarum 965; 965; L. cellobiosus, LC; and natural fermentation, N. Cover brines were not supplemented with malic acid.

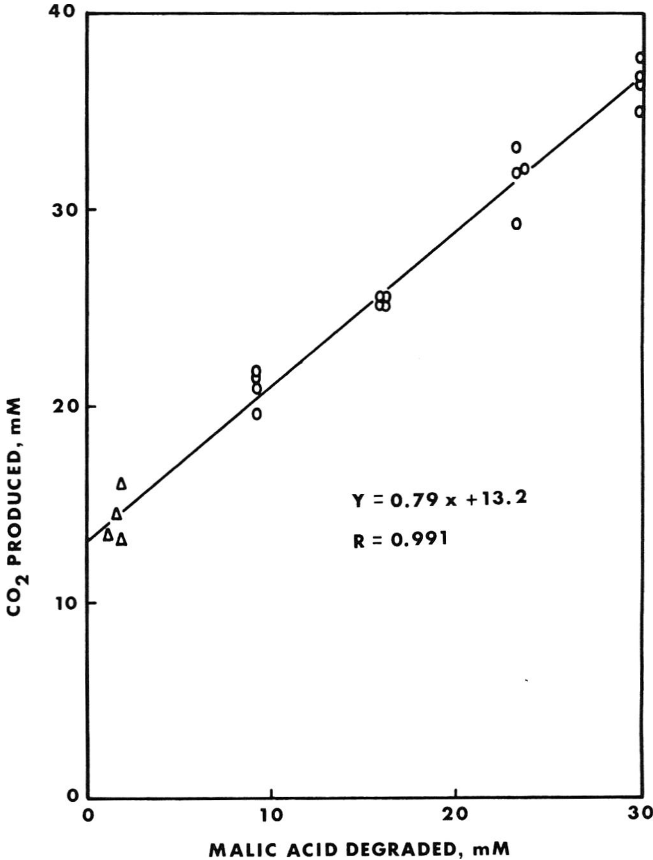

Fig. 2–Relationship between malic acid degradation and CO_2 formation in cucumber fermentations: △ – L. plantarum 965 without malic acid added to the cucumbers; ○ – L. plantarum WSO with 0, 7, 14, and 21 mM malic acid added to the cucumbers.

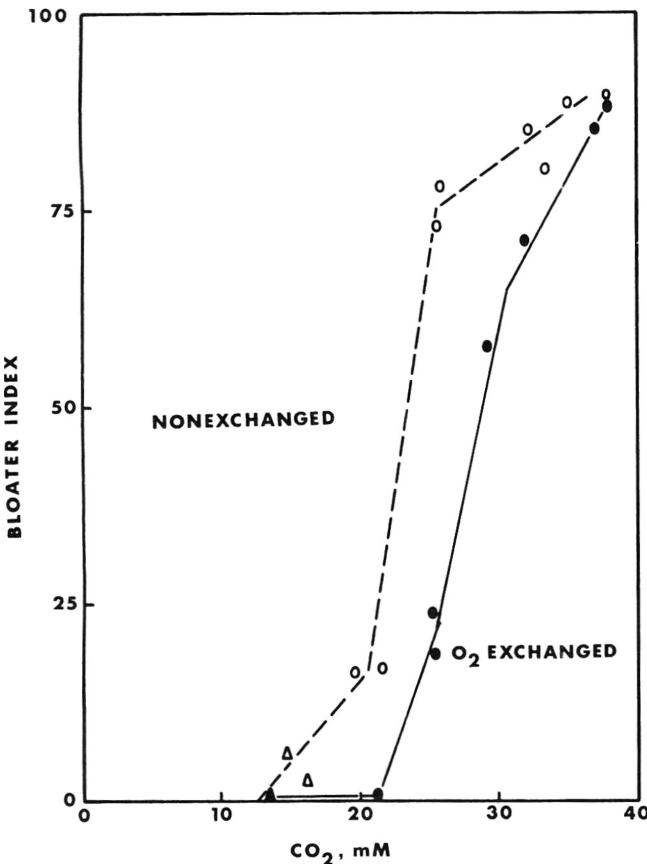

Fig. 3—Effect of O_2 exchange on bloater damage of fermented cucumbers: △, ▲ – L. plantarum 965 without malic acid added to the cucumbers; ○, ● – L. plantarum WSO with 0, 7, 14, and 21 mM malic acid added to the cucumbers.

(1983) indicate that inability to degrade malic acid is not a common characteristic among this group of bacteria. However, Daeschel et al. (1984) have developed a rapid selection procedure for nonmalic acid-degrading lactic acid bacteria and have demonstrated the isolation of such mutants from *L. plantarum* WSO. Therefore, it may be possible to obtain low CO_2-producing organisms with better fermentation characteristics than the *L. plantarum* 965 strain now available.

SUMMARY

TWO STRAINS of *L. plantarum* (963 and 965) from 24 strains of *L. plantarum* and *P. cerevisiae* tested did not degrade all malic acid from a test medium. *Lactobacillus plantarum* 965, which degraded the least malic acid, prevented significant bloating of cucumbers by reducing the amount of CO_2 produced during fermentation in comparison with *L. plantarum* WSO, which completely degraded malic acid.

The total CO_2 production during a fermentation could be divided into two parts. CO_2 from sources other than malic acid, primarily cucumber tissue metabolism, amounted to 12.5 mM. This concentration of CO_2 was sufficient to bring the cucumbers to the critical point above which measurable bloating damage would occur. CO_2 formation above 12.5 mM was directly related to the amount of malic acid degraded. This additional CO_2 provided the marginal increase in gas production required to cause bloater damage. Bloating damage increased with the increase in CO_2 formation from malic acid degradation.

Previous work had shown that oxygen exchange of cucumbers prior to brining increased their resistance to bloating during fermentation (Fleming and Pharr, 1980). Comparison of exchanged and nonexchanged cucumbers showed that exchange O_2 increased the concentration of CO_2 required to initiate bloating by 8 mM.

These results indicate that fermentation of cucumbers with nonmalic acid-degrading starter cultures and/or use of oxygen exchange procedures may make it possible to prevent bloater damage in cucumbers without the need to remove CO_2 from the brines by purging (Fleming, 1979).

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Determination of Equivalent Processes for the Pasteurization of Crabmeat in Cans and Flexible Pouches

D. R. WARD, M. D. PIERSON, and M. S. MINNICK

ABSTRACT

Equivalent processes were determined for the pasteurization of crabmeat, from the blue crab (*Callinectes sapidus*), in cans and flexible pouches containing 113.5g, 227.0g, and 454.0g of product. F_{185} values were calculated for z values of 8, 10, 12, 14, 16, 18, 20, and 22. Based on the heating characteristics of the traditional container used for pasteurized crabmeat (401 x 301), equivalent processes for nontraditional containers were determined.

INTRODUCTION

THE PROCESS of pasteurizing meat from the blue crab (*Callinectes sapidus*) was first developed by Anzulovic and Reddy (1942); however, the process was refined and patented by Byrd (1951). Byrd's patent covered pasteurization temperatures from 77° - 99°C). Tatro (1970) published a guideline of 85°C for 1 min for the pasteurization of crabmeat. Tatro's work was done with 401 x 301 cans holding 16 oz (454g) of crabmeat. According to the guideline, 110 - 115 min in an 87.8° - 88.9°C water bath is usually needed to achieve 85°C for 1 min at the cold point of can. Furthermore, Tatro (1970) recommended immediately cooling the crabmeat to 37.8°C in an ice water bath prior to refrigerated storage.

Most processors of pasteurized crabmeat have adhered to Tatro's (1970) guidelines and have successfully produced a pasteurized product in 401 x 301 cans. In recent years, however, several factors have prompted the industry to evaluate can sizes other than the 401 x 301, as well as containers other than metal cans. Some of these factors are; a 1-lb can of crabmeat is relatively expensive; therefore, in order to reduce the purchase price they have reduced the amount of product which must be purchased; the cost of metal cans has made plastic containers of interest to the industry; and the energy efficiency of retortable pouches has also been noted with interest within the industry. Developing processes for new containers, however, presents problems. The guidelines are not based on inactivation of specific microorganism(s) or enzymes; rather they are based on the production of a product with 6-month refrigerated shelf-life that has acceptable sensory attributes. There is currently no recommended process for containers other than the 401 x 301 can.

The purpose of this work was to evaluate the F_{185} for different z values by the thermal process traditionally used in the pasteurization of crabmeat, and to determine equivalent processes for nontraditional can sizes and containers.

MATERIALS & METHODS

CANS USED for processing 113.5g and 227.0g of crabmeat were 211 x 114 and 307 x 206, respectively. Both 401 x 301 and 303 x 406 cans were used to process 454.0g of crabmeat. Flexible pouches were a three-ply laminate consisting of an outer layer of polyester, a middle layer of aluminum and an inner layer of polypropylene (Reynolds Metals Co., Richmond, VA). The pouch dimensions for 113.5g, 227.0g, and 454.0g of crabmeat were 12.7 x 11.4 x 2.6 cm, 12.7 x 12.6 x 3.4 cm, and 17.8 x 14.6 x 4.5 cm, respectively.

Internal temperatures of crabmeat in cans were measured during processing with rigid type T (copper constantan) molded bakelite thermocouples (O. F. Ecklund, Cape Coral, FL). Thermocouples were installed with the temperature-sensitive tip located at the geometric center of the can. Internal temperatures of crabmeat in flexible pouches were measured using 24 gauge nylon insulated, copper-constantan thermocouple wire (O. F. Ecklund). Flexible pouches were prepared for thermocouple installation by cutting an 8 mm diameter hole in the side seam. Thermocouple wire lengths were inserted into the pouch to the geometric center, a stuffing box (O. F. Ecklund) consisting of brass fittings and rubber gaskets was used to seal each thermocouple in the pouch. Pouches were placed vertically into compartments of wire-mesh racks to control pouch thickness during pasteurization. These compartments were configured such that the wire-mesh racks fit snugly against both sides of the filled pouches. Although no attempt was made to measure the dimensions of the pouch during heating, it was assumed that the thickness of the pouch was adequately controlled during heating using this procedure.

Pasteurization was done in a steam-heated water bath preheated to 87.8°C. Packaged crabmeat was heated to an internal temperature of 85°C for 1 min and immediately cooled in an ice water bath (0°C) to less than 10°C within 1 hr. Thermocouples were monitored during heating and cooling; all data were recorded at 1-min intervals (Monitor Labs, Inc., Data Logger, model 9300).

Process lethalities were obtained using time-temperature data from the various processed containers. Based on the "improved" general method, temperatures were converted to equivalent lethal values and summed together to obtain F values (Patashnik, 1953).

RESULTS & DISCUSSION

FIG. 1 gives the heating and cooling curves for 454g, 227g, and 113.5g containers at the cold point. Cold points for all containers were experimentally determined. These curves are composite curves derived from statistical interpolation of 8 replicates of each container size. Additionally, Table 1 shows the heating and cooling parameters for the various containers. Fig. 2 shows the process lethalities (F_{185}) at different z values for the various containers.

The 401 x 301 can containing 454g of crabmeat is the container from which Tatro (1970) derived the data which led to the publication of the original guidelines for the pasteurization of crabmeat. Furthermore, this container has traditionally been the container of choice by most of the pasteurized crabmeat industry (Nelson, 1983) and by using this container according to Tatro's (1970) recommendations crabmeat has been safely and successfully marketed. Therefore, the 401 x 301 container and Tatro's process can be considered as the "standard" for crabmeat pasteurization. If a z value of 16 is assumed for the standard 401 x 301 container and process, the resulting equivalent process time at 85°C (185°F) is 31 min. Should a processor desire to use a 303 x 406 can, containing 454.0g of crabmeat according to the data presented in Fig. 2, once the cold point reaches 85°C the processor must hold the product for an additional 7 min in order to achieve an $F_{185}^{16} = 31$, which would then be equivalent to the "standard" process. Similarly, the 454.0g pouch, the 227.3g (307 x 206) can,

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the 227.9g pouch, the 113.5g (211 x 114) can and the 113.5g pouch would need to be held for 14, 15, 22, 20, and 24 min, respectively. Although additional holding time at 85°C would be required for containers to achieve an F_{185}^{16} = 31, the total processing time (heating and cooling) would be reduced. For example, the standard 401 x 301 can required 145 min total process time to heat to 85°C, hold for 1 min, and then cool to 37.8°C (Fig. 1A). On the other hand, the 454.0g pouch and 303 x 406 can required a total process time of 118 min and 135 min, respectively; these times included the 14 min (pouch) and 7 min (303 x 406 can) additional holding at 85°C required to achieve a process lethality equivalent to the "standard" process. Total processing time required to achieve an equivalent process in the 227.0g pouch was 75 min and 109 min in the 307 x 206 can (Fig. 1B). Additionally, the 113.5g pouch required 62 min and the 211 x 114 can required 77 min (Fig. 1C). Should F values be required with z values other than the 16 used in these examples, Fig. 2 provides a basis for their calculations.

Very little information regarding the lethality of pasteurized crabmeat has been published. Dickerson and Berry (1974) evaluated the lethality of a crabmeat pasteurization process in Virginia to be equivalent to an F_{180}^{14} = 18 for a 401 x 301 can. Lynt et al. (1977) reported that this process level was considerably above that required to destroy 10^5 - 10^6 spores of *Clostridium botulinum* type E. Lynt et al. (1977) reported that the D value at 85°C for the Beluga strain of *C. botulinum* type E was 0.29 (upper value = 0.41, lower value = 0.16); the authors went on to state, however, that 1 min at 85°C did not appear to be sufficient to destroy all spores of type E in crabmeat. This latter statement has caused some confusion among state control agencies

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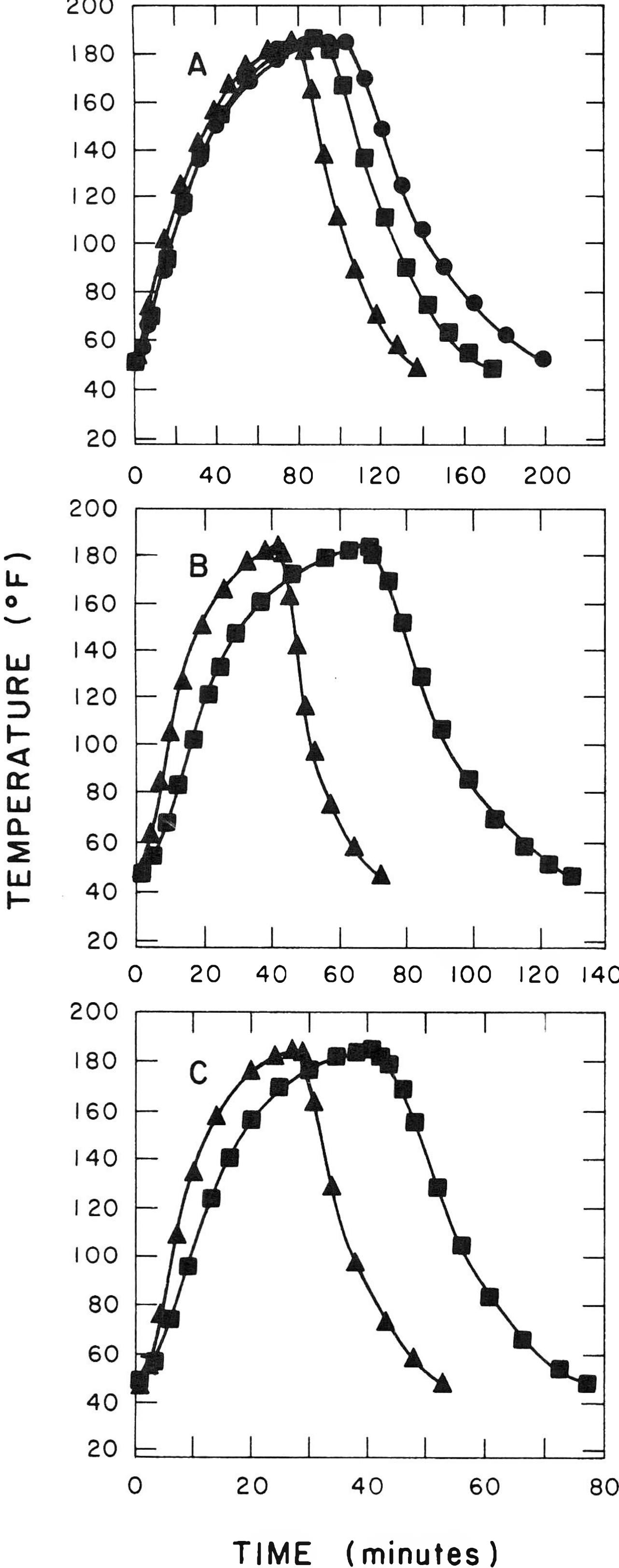

Fig. 1—Heating and cooling curves for (A) 454.0g crabmeat in pouches (▲) and 303 x 406 (■) and 401 x 301 (●) cans. (B) 227.0g crabmeat in pouches (▲) and 307 x 206 cans (■); (C) 113.5g crabmeat in pouches (▲) and 211 x 114 (■) cans; pasteurized to 85°C (185°F) for 1 min.

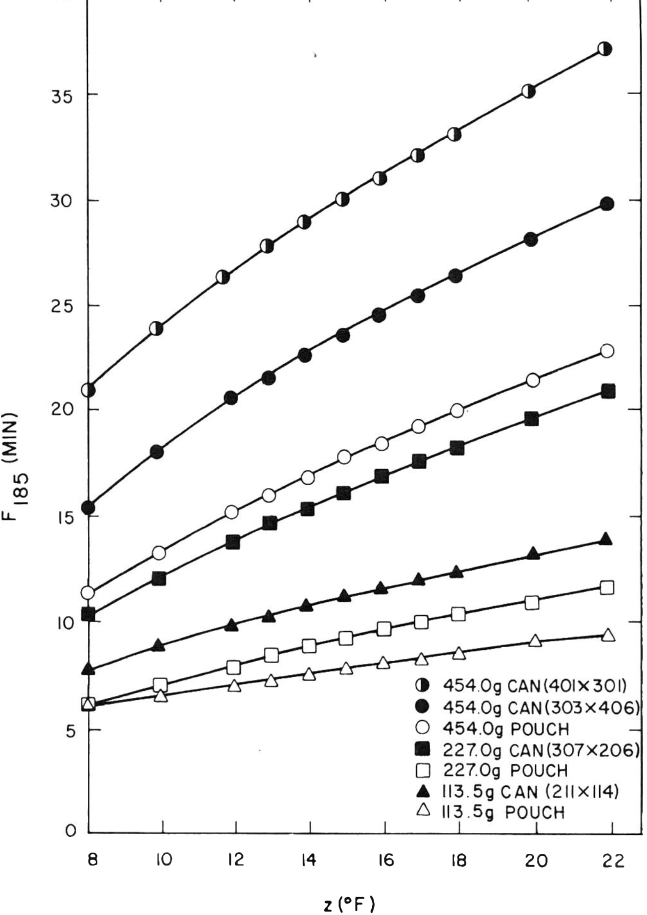

Fig. 2—F values for containers pasteurized to 85°C (185°F) for 1 min and subsequently cooled to less than 10°C (50°F).

Manufacture and Quality of Venezuelan White Cheese

IVELIO ARISPE and DENNIS WESTHOFF

ABSTRACT

A survey of the manufacture of queso blanco, the most popular cheese in Venezuela, confirmed a lack of standardization. Based on compositional factors, four desired cheese types were established and prepared in a pilot plant. Significant ($p < 0.05$) variation in moisture, salt, fat and protein were noted. During storage at 15°C for up to 8 days, variation in cheese composition including an increase in acidity was also significant ($p < 0.05$). Increases in acidity were not high enough to prevent growth of native Staphylococci, fecal coliforms, and lactobacilli.

INTRODUCTION

VENEZUELAN QUESO BLANCO is considered the most popular cheese in the country. In spite of its high consumption and increased production in Venezuela, several researchers (Escoda and Hernandez, 1968; Boscan, 1978) have shown that there does not exist defined, standardized commercial manufacturing procedures for queso blanco production. Presently there are many different types of this cheese, with a high percentage still made at the farm level.

A survey of the Venezuelan market (Arispe and Westhoff, 1983) showed that compositional factors (including fat, protein, salt and moisture) varied significantly. Microbiological findings suggested that considerable improvement could be made in the manufacture and handling of queso blanco. The current research was conducted: (1) to study the actual commercial manufacturing conditions of Venezuelan queso blanco; (2) to standardize a pilot plant manufacturing procedure; and (3) to study the effect of process variables on cheese quality.

MATERIALS & METHODS

Survey of the industrial procedures for queso blanco manufacture

A questionnaire was completed to provide a description of the processing conditions at each of several important cheese plants located in the West and Northwest of Venezuela. Additionaly, quality control parameters were determined in collaboration with plant personnel. These results were analyzed and used as the basis for experimentation in our pilot plant.

Pilot plant process and manufacture

Moisture and salt contents are considered the most important compositional factors related to the microbial content and stability of cheese. Using the information obtained regarding these parameters from industrial processors, several queso blanco manufacture procedures were designed. Fig. 1 outlines the pilot plant operation used initially. Fourteen vats of cheese were made to ensure that the selected procedures would result in a product with the desired moisture and salt concentration.

Experimental variables studied included: rennet, calcium chloride and sodium chloride concentrations; time and form of cutting, stirring and treatment of the curd in the vat, time of draining and volume of the first whey, time and method of salting, and method and characteristic of pressing (Fig. 1).

All cheese was made in 30-liter stainless steel vats. The vats, knives, and curd shovels were constructed by the E.A. Kastner Company (Baltimore, MD). The pressing operation was done in a stainless steel perforated mold, with 0.5 cm diameter holes and total volume of 5,625 cm^3 (25 cm x 15 cm x 15 cm). Pressure was obtained using 10, 20, and 30 kg weights, resulting in pressures of approximately 0.044, 0.088, and 0.133 kg/cm^3, respectively. The loss of moisture, weight and the yield under each condition was determined.

As a result of preliminary experiments, 0.15 g/L of CaCl and 0.025 g/L of rennet was considered suitable for a firm curd in 35 min at 32°C. To standardize the salting procedure a total whey volume equal to 75% of the milk was drained, followed by the addition of 15% salt whey brine solution. A combination of agitation for 10 min and a hold of 20 min or agitation for 1 min and hold for 4 min was necessary to obtain a concentration of 3% and 2% salt, respectively, in the fresh cheese.

Monitoring of pilot plant processes

Information obtained from the processors' survey indicated that

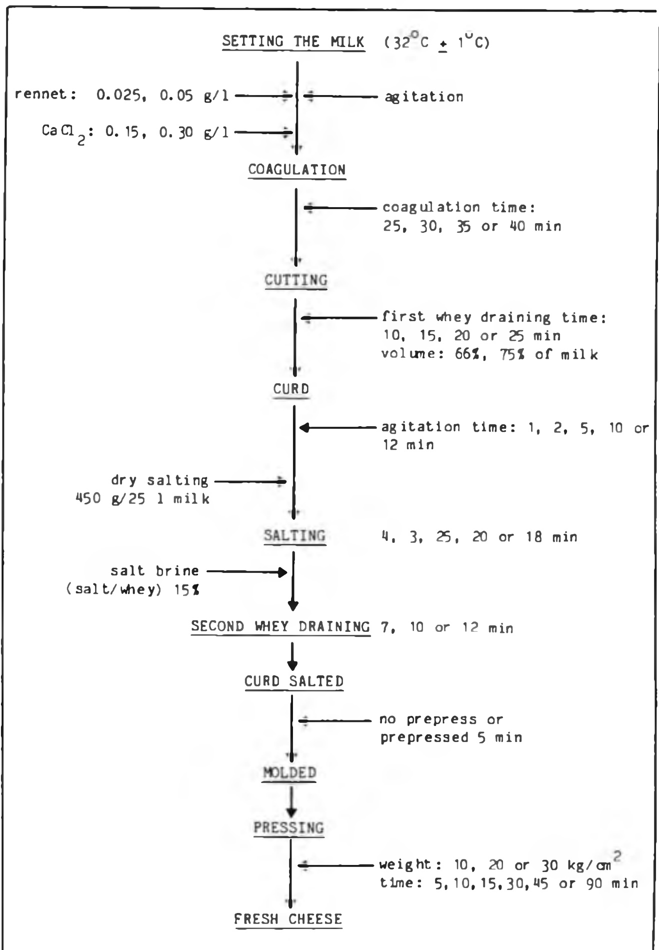

Fig. 1—Schematic of the queso blanco pilot plant procedures used in the preliminary experiments.

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four variations should be selected for evaluation. A total cf 12 vats of cheese were made, 3 of each of the following types: (1) high moisture (59 ± 1%), low salt (1.5 - 2.2% NaCl); (2) high moisture (59 ± 1%), high salt (3 - 3.5% NaCl); (3) low moisture (52 ± 1%), low salt (1.5 - 2.2% NaCl); and (4) low moisture (52 ± 1%), high salt (3 - 3.5% NaCl).

Cheese analysis

Cheese types were stored at 3°C and 15°C for up to 8 days. During various stages of manufacture and storage, the following parameters were monitored: moisture, fat, total protein, salt, acidity and pH (Kosikowski, 1977), total and fecal coliforms, *Staphylococcus aureus* (AOAC, 1978) and lactobacilli (Mean et al., 1960).

Statistical analysis

Two-way analysis of variance (Snedecor and Cochran, 1967) and a multiple comparison (Duncan, 1955) were performed.

Table 1—Comparison of three commercial manufacturing procedures for Venezuelan queso blanco

	Commercial Cheese Plants		
Step	A	B	C
Milk			
Pasteurization	77°C – 15 sec.	77°c – 18 sec.	72°C – 20 sec.
Acidity (%)	0.17	0.16 – 0.22	0.18 – 0.20
Fat (%)	2.8 – 4.4	3.2	4.3 – 4.4
Standardization (Fat %)	4.4	3.8	2.8
Vat			
Size (liters)	4,000	4,600	2,300
Inoculating temperature (°C)	35-36	32	36
Time to reach temp (min)	35-40	35	30
Coagulation			
$CaCl_2$ (g/1,000L)	150	152	87
Rennet (per 100oL)	20g	20g	26g
Coagulation time (min)	35	35	25
Cutting			
Method	Mechanical	Mechanical	Manual
Agitation (min)	15	8	9
First draining			
Time (min)	20	44	40
Salting			
Method	spreading	immersion	spreading
Amount (g/L)	18,8	75	32.6
Second draining			
Prepress?	yes	no	no
Time (min)	15	10	25
Pressing			
Type force	hydraulic	weights	hydraulic
Amount of force	?	20 kg/400 cm^2	?
Storage			
Time (days)	2	2	2
Temperature (°C)	5	5	5
Yield (kg/100L)	13.5	14	15
Total process (hr:min) without storage	3:12	3:10	3:24
Characteristics of cheese			
NaCl (%)	2.5	3.0	2.6
Moisture (%)	52-54	55-56	55-58
Fat (%)	23	16	19
pH	6.1	6.0	6.1

RESULTS & DISCUSSION

Survey of current methods of queso blanco manufacture

Table 1 summarizes the information collected at three major queso blanco producing plants coded as A, B and C. These plants manufacture mainly a soft queso blanco with a high moisture content, ranging from 54 - 58%. The coagulation process was essentially enzymatic and no starter cultures were used. All of the plants had high temperature-short time (HTST) pasteurizing systems. Normally, the three plants held the fresh cheese about 2 days at 5°C before salt, 1 day at the plant and another in refrigerated distribution by truck.

The variation in the process used in these plants and information collected in two smaller cheese plants confirmed the lack of standaridzed procedure for the manufacture of queso blanco (Escoda and Hermandez, 1968; Boscan, 1978). The wide compositional variation and quality currently found in this cheese (Arispe and Westhoff, 1983) might be attributed to these processing differences.

Pilot plant manufacture of queso blanco

Fig. 2 illustrates the moisture lost during different pressing conditions. The decrease in moisture was rapid only during the first 15 min. Prolonged pressing, up to 75 min, had minimal effect. In order to obtain a lower moisture (about 52%) in the fresh cheese, pressure was applied for 5 min to the curd in vat. Starting with 63% curd moisture and 30 kg/225 cm^2 pressure for 75 min, a fresh cheese moisture of 52% was obtained (Fig. 2). Corresponding weight losses for these same pressing conditions are shown in Fig. 3.

The procedure outlined in Fig. 4 permitted the manufacture of four different types of soft queso blanco with the following characteristics: (1) relatively high moisture (59 ± 1%) low salt content (1.5 - 2.2%) cheese, total manufacture time of 107 min; (b) high moisture (59 ± 1%), high salt (3 - 3.5%), 132 min; (c) relatiely low moisture (52 ± 1%), low salt (1.5 - 2.2%), 192 min; and (d) relatively low moisture (52 ± 1%), high salt (3 - 3.5%), 217 min.

The manufacture procedure for queso blanco differs from other Latin American white cheese (Siapantas and Kosikowski, 1967; Chandan and Marin, 1979). For example, coagulation occurs at relatively low temperature

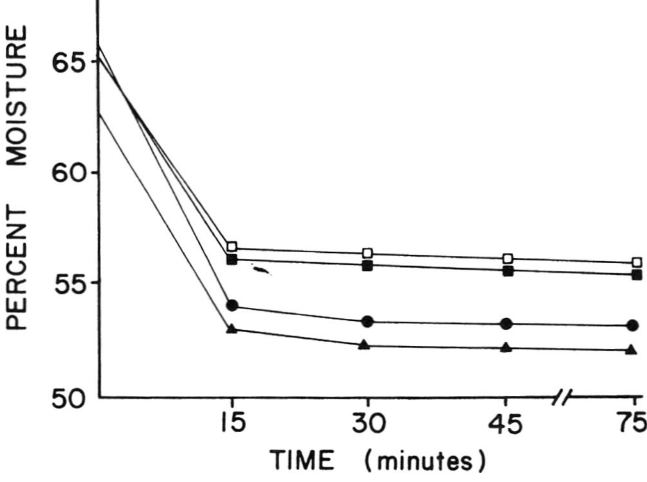

Fig. 2—Loss of moisture from Venezuelan queso blanco during pressing: (□ – □) 10 kg: 0.044 kg/cm^2; (■ – ■) 20 kg: 0.089 kg/cm^2; (● – ●) 30 kg: 0.133 kg/cm^2 plus 5 min of prepressure to the curd in the vat; (▲ – ▲) 30 kg: 0.133 kg/cm^2.

(32°C) and rennet is used. Additionally, queso blanco has a very short shelf life and possesses a unique taste.

The curd and cheese yields obtained by the pilot plant procedures are summarized in Table 2. Starting with 100 liters of milk about 17.8 kg of curd could be obtained, which yielded approximately 13.5 kg fresh cheese. Composition of the milk, the moisture content of the final cheese, and the degree of recovery of fat and casein by the curd are considered the three most important factors influencing cheese yield (Kosikowski, 1977). Chandan and Marin (1979) reported an average yield value of 11.6% for Latin American white cheese. These yield values were dependent on the type of acid used as coagulant. Kosikowski (1977) suggested that an average yield for cheddar cheese would be about 9.5%.

Cheese analysis

The moisture, acidity, salt, fat, and protein contents, and the pH in four different types of cheese were evaluated. Additionally, the cheese was stored at 3°C and 15°C for up to 8 days. The different manufacturing conditions produced significant ($p < 0.05$) variation in cheese moisture, salt, fat and protein contents (Table 3). Cheese composition was also significantly ($p < 0.01$) affected by storage. There was a significant ($p < 0.05$) interaction between moisture and type of cheese made (relatively high moisture and relatively low moisture). When each of these parameters was considered separately during storage (Table 4), the decrease in moisture was significant ($p < 0.05$) for high moisture cheese. For relatively low moisture cheese, the decrease in moisture was only significant following storage on 8 days at 15°C. The variation in salt content and the corresponding statistical analyses is presented in Tables 3 and 4. For each type of cheese, decreasing moisture was all types of cheese, a significant ($p < 0.05$) salt content increase was observed during storage for 8 days at 15°C. This increase was also significant for relatively low salt cheese when stored at 3°C for 8 days. The increase in salt content was the result of the moisture loss during cheese storage.

The moisture content of cheese is directly related to its nutritional, biological and survey (Van Slyke and Price, 1949). The higher the moisture content, the lower the concentration of nutrients. For economic reasons, the cheese industry in Venezuela encourage regulations that could permit higher moisture cheese. The Venezuelan Regulatory Agency and the consumer are concerned with the variation and the stability of the same cheese in the market. There is a significant relationship (Arispe and Westhoff, 1983) between the microbial quality and the moisture content of market quaso blanco.

The salt content is one of the most important factors influencing ripening and final quality of cheese (Davis, 1965; Pearce and Glies, 1979). The ratio of salt to moisutre (3W) should be between 4.2 and 5.2. Salt in cheese also has the function of suppressing the growth of unwanted organisms. In addition, lactose utilization and lactic acid production by starter bacteria are affected by variation in salt to moisture (S/M) in cheddar cheese (Turner and Thomas, 1980).

Table 2—Yields of curd and cheese during the pilot plant manufacture of Venezuelan queso blanco

	Yield (kg/100 liters of milk)	
	Curd[a]	Cheese[b,c]
$\bar{X}$	17.8	13.5
Range	15.3 - 20.2	10.5 - 15.3
Standard deviation	1.8	1.6
Coefficient of variation (%)	9.9	11.5
Number of samples	14	14

[a] Curd moisture averaged 66.3%.
[b] Cheese moisture averaged 55.0%.
[c] Fat content averaged 24.0%.

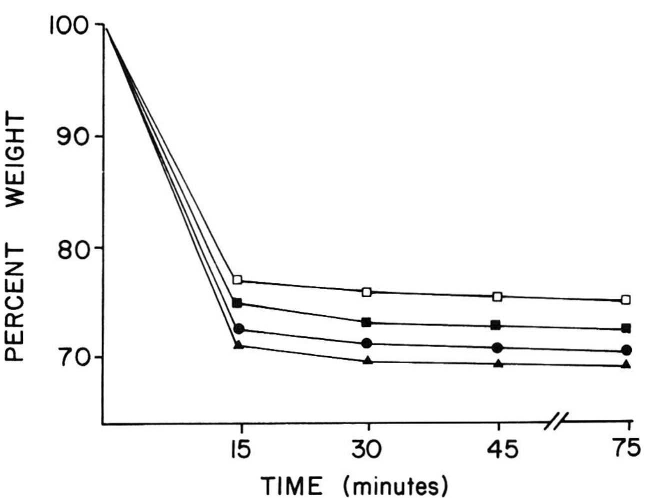

Fig. 3—Decrease in weight of Venezuelan queso blanco during pressing: (□ – □) 10 kg: 0.044 kg/cm²; (■ – ■) 20 kg: 0.089 kg/cm²; (● – ●) 30 kg: 0.133 kg/cm² plus 5 min of prepressure to the curd in the vat; (▲ – ▲) 30 kg: 0.133 cm².

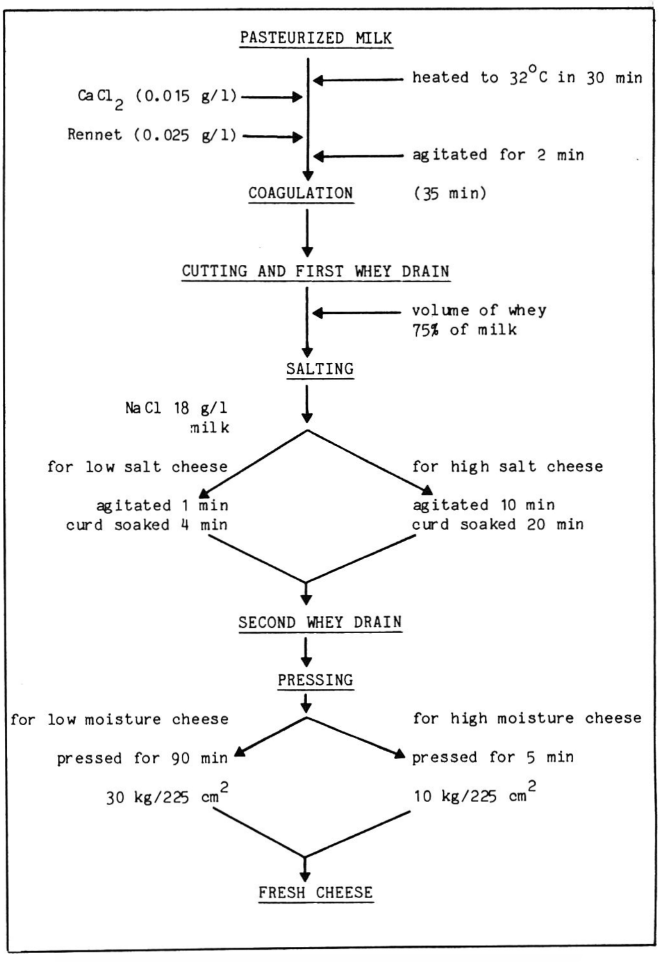

Fig. 4—Schematic of the queso blanco plant procedures used to produce the experimental samples.

The loss of moisture during storage resulted in an increase of the salt concentration by 25% at 3°C and 33% at 15°C (Table 4). The consequence of the loss of moisture in cheese suggests the need to regulate the salt content in fres queso blanco and to define and standarize the proper moisture level for the soft Venezuelan queso blanco.

During cheese ripening, a small amount of neutral fats are hydrolyzed to butyric, caproic, caprylic and low chain fatty acids. These are known to contribute mainly to the development of a more aromatic flavor of cheese. Queso blanco is an unripened cheese, therefore the fats contribute mostly to the nutritional and textural characteristics of the cheese. Fat content variation in queso blanco stored at different conditions is presented in Table 4. Low moisture cheese had more fat than high moisture cheese and as a consequence of moisture loss during storage, the fat concentration increased more than in high moisture cheese. This was also true for the protein content (Table 4). In addition, there were low levels of soluble proteins which did not change significantly during 8 days of storage (data not shown).

The different manufacturing conditions did not produce significant variations in pH and acidity (Table 4). The variations observed were due to the time and temperature of storage. The higher variations were mainly produced in cheese stored for 8 days at 15°C. A significant ($p < 0.05$) increase in pH was observed during 8 days of storage at 3°C and at 15°C.

The coagulation activities of rennet vary with the acidity of the milk and the acidity produced by the lactic starter bacteria. The rate of acid development depends on the type starter and on the milk composition. The acidity produced influences the texture, taste and flavor during subsequent ripening of the cheese. Acidity also helps to control unwanted microorganisms. Acidity produced during the cheese making has been studied by several authors (Brown and Price, 1934; Price et al., 1971; Olson, 1976). Desired changes in titratable acidity and pH values during the manufacture of good quality cheddar cheese have been established (Price et al., 1971). A pH between 5.0 – 5.3 in 1 day old cheese was sufficient to produce a good cheese. In queso blanco manufacture, starting with a 0.19% titratable acidity in the milk, the acidity in the whey is usually 0.12%. During manufacture, no significant increase ($p > 0.05$) of acidity in the whey after 2 hr was observed. A significant ($p < 0.05$) increase in acidity occurred during storage for 8 days (Table 4), which was attributed to the growth of the native lactic microflora.

Table 3—Statistical summary (two-way analysis of variance) of the effect of manufacturing conditions on queso blanco composition

	Compositional factors					
	Moisture	pH	Acidity	NaCl	Fat	Protein
Treatment (T)[a]	0.001*	0.368	0.154	0.001*	0.001*	0.002*
Storage conditions (S)[b]	0.001*	0.001*	0.001*	0.003*	0.001*	0.004*
Interaction (TXS)	0.05**	0.124	0.124	0.990	0.990	0.990

[a] Type of cheese: Type I, moisture 59 ± 1%, salt 1.5 – 2.2%.
Type II, moisture 59 ± 1%, salt 3.0 – 3.5%.
Type III, moisture 52 ± 1%, salt 1.5 – 2.2%.
Type IV, moisture 52 ± 1%, salt 3.0 – 3.5%.

[b] Storage conditions were 0, 2, 5 and 8 days at 3°C and 5 and 8 days at 15°C.
*Differences are significant at $p < 0.01$.
**Differences are significant at $p < 0.05$.

Table 4—Variation in moisture, salt, fat, protein, pH and acidity during storage of the four types of experimental Venezuelan queso blanco (mean values)

		Days (d) and temperature of storage					
	Type of	0 day	2 days	5 days	8 days	5 days	8 days
Parameter	cheese[c]		3°C	3°C	3°C	15°C	15°C
Moisture (%)	I	59[a]	58.1	56.5	55.1[b]	56.3	53.8[c]
	II	58.7[a]	57.9	56.4	55.8[b]	55.3	53.6[c]
	III	53.1[a]	54.1	53.2	51.6[a]	52.8	50.2[b]
	IV	52.9[a]	54.4	53.3	52.1[a]	52.5	50.2[b]
NaCl (%)	I	1.9[a]	2.1	2.8	2.4[b]	2.5	2.5[c]
	II	3.0[a]	3.1	3.1	3.2[a]	3.2	3.3[b]
	III	2.1[a]	2.2	2.4	2.4[b]	2.4	2.5[b]
	IV	3.3[a]	3.1	3.1	3.4[a]	3.5	3.6[b]
Fat (%)	I	23.5	23.6	23.8	25.2[a]	25.5	26.8[a]
	II	23.5[a]	24.8	24.5	24.8[a]	27.1	27.5[a]
	III	25.5[a]	26.8	28.6	28.3[b]	27.0	30.0[b]
	IV	24.7[a]	27.5	27.5	29.0[b]	28.3	30.0[b]
Protein (%)	I	16.9[a]	17.4	17.2	17.2[a]	18.9	18.5[a]
	II	16.4[a]	17.1	17.5	17.9[a]	17.9	19.2[a]
	III	18.5[a]	18.6	17.6	19.0[a]	17.9	19.9[a]
	IV	18.4[a]	18.2	19.8	19.8[a]	19.2	21.5[b]
pH	I	6.6	6.5	6.5	6.41[a]	6.4	6.2[b]
	II	6.5[a]	6.5	6.5	6.4[a]	6.4	6.3[a]
	III	6.5[a]	6.5	6.5	6.4[a]	6.5	6.5[a]
	IV	6.5[a]	6.5	6.5	6.4[a]	6.5	6.42[ab]
Acidity (%)	I	0.06[a]	0.12	0.11	0.12[b]	0.12	0.15[c]
	II	0.06[a]	0.11	0.10	0.11[b]	0.12	0.13[b]
	III	0.09[a]	0.10	0.11	0.11[b]	0.13	0.15[c]
	IV	0.07[a]	0.10	0.10	0.12[b]	0.10	0.13[b]

[a,b] Superscripts which share a common letter within each treatment indicate that they were not significantly different ($p > 0.05$); however, only 8 day values were compared to day zero values.
[c] Type cheese — See footnote to Table 3.

Monitoring the native microflora

The growth of staphylococci, total coliforms, fecal coliforms and lactobacilli in queso blanco during various stages of manufacture and storage was determined. Initial numbers of each microbial group were significantly different between vats ($p < 0.05$), therefore $\log_{10}$ counts were converted to percent so the initial count in the milk for each group and replications was considered as 100%. Subsequent counts were expressed as percent of change from the initial counts.

The different cheese manufacturing conditions produced a highly significant ($p < 0.01$) effect on the growth of staphylococci and total coliforms (Tables 5 and 6). Additionally, growth of staphylococci, total and fecal coliforms and lactobacilli was significant ($p < 0.05$) during storage for 8 days (Tables 7 and 8).

During the manufacturing process, from the milk to zero days of storage for all the queso blanco types, there was an increase of at least one log cycle in the number of staphylococci (Table 5). Although some microorganisms were removed in the whey, many of them were concentrated during the curd formation (Takahashi and Johns, 1959; Thatcher and Ross, 1960). The observed increase was significant ($p < 0.05$) for the low moisture cheese (Table 5). This could be due to a longer pressing time and consequently longer manufacture time (220 min) compared to 135 min for the high moisture cheese types. Growth of staphylococci was not significant ($p > 0.05$) for all types of cheese when they were stored at 3°C for up to 8 days. However, when the low moisture cheese types were stored at 15°C, there was an increase in growth, which was significant ($p < 0.05$) (Table 5). The significantly higher growth observed in the low moisture cheese as compared to the high moisture can be attributed to the longer manufacturing time and also to a significant difference ($p < 0.05$) in

Table 5—Fate of Staphylococci during manufacturing and storage of the four experimental types of Venezuelan queso blanco

Cheese type[e] (Treatment)		Stages of manufacture				Days (d) and temperature (°C) of storage					
		Milk	Whey	Curd	Salted curd	0 day	2 days 3°C	5 days 3°C	8 days 3°C	5 days 15°C	8 days 15°C
I	Log10[f]	4.00	2.92	4.86	4.85	5.00	5.06	5.12	5.51	6.08	6.86
	Percent[g]	100[a4]	73	121	121	125[ab]	126[ab]	127[ab]	137[ab]	152[ab]	171[b]
II	Log10	4.00	2.73	3.74	4.05	4.07	4.08	4.32	4.60	5.35	6.60
	Percent	100[a]	68	93	101	101[a]	102[a]	106[a]	115[a]	134[ab]	165[b]
III	Log10	1.36	1.94	2.24	2.86	3.25	4.13	4.26	4.63	6.13	7.01
	Percent	100[a]	166	205	237	257[b]	359[bc]	349[bc]	359[bc]	469[d]	540[d]
IV	Log10	1.36	1.99	2.77	3.02	3.30	4.14	4.58	4.51	5.06	6.36
	Percent	100[a]	119	215	231	247[b]	320[bc]	357[bc]	343[bc]	337[bc]	490[d]

[a–d] Superscripts which share a common letter within each treatment indicate that they were not significantly different ($p > 0.05$).
[e] Cheese type — See footnote to Table 3.
[f] Mean of counts ($\log_{10}$/g).
[g] Assuming mean count in milk to be 100% other counts expressed as the percentage of initial count.

Table 6—Fate of coliforms during manufacturing and storage of the four experimental types of Venezuelan queso blanco

Cheese type[d] (Treatment)		Stages of manufacture				Days (d) and temperature (°C) of storage					
		Milk	Whey	Curd	Salted curd	0 day	2 days 3°C	5 days 3°C	8 days 3°C	5 days 15°C	8 days 15°C
I	Log10	3.13	2.29	4.80	4.69	5.02	4.61	5.26	4.73	5.24	6.28
	Percent[f]	100[a]	210	214	192	181[ab]	203[ab]	207[ab]	224[ab]	244[ab]	296[ab]
II	Log10	3.13	1.60	4.31	3.46	3.91	2.92	2.13	3.11	3.90	4.91
	Percent	100[a]	72	214	165	178[a]	143[a]	150[a]	186[a]	199[ab]	257[b]
III	Log10	1.20	1.75	2.50	2.13	3.13	2.65	2.74	3.13	3.34	4.38
	Percent	100[a]	80	210	223	270[b]	245[b]	241[b]	268[b]	279[b]	383[c]
IV	Log10	1.20	1.90	2.65	2.22	2.02	2.24	2.49	2.03	2.88	2.50
	Percent	100[a]	110	232	197	179[a]	184[a]	191[a]	210[a]	224[b]	263[b]

[a–c] Superscripts which share a common letter within each treatment indicate that they were not significantly different ($p > 0.05$).
[d] Cheese type — See footnote to Table 3.
[e] Mean of counts ($\log_{10}$/g).
[f] Assuming mean count in milk to be 100% other counts expressed as the percentage of initial count.

Table 7—Fate of fecal coliforms during manufacturing and storage of the four experimental types of Venezuelan queso blanco

Cheese type[b] (Treatment)		Stages of manufacture				Days (d) and temperature (°C) of storage					
		Milk	Whey	Curd	Salted curd	0 day	2 days 3°C	5 days 3°C	8 days 3°C	5 days 15°C	8 days 15°C
I	Log10	2.98	1.92	3.48	3.50	3.91	3.89	3.17	2.63	4.71	4.06
	Percent[d]	100[a]	98	143	139	107[a]	191[a]	167[a]	175[a]	133[a]	235[a]
II	Log10	2.98	0.65	2.44	2.09	2.00	1.45	1.20	1.20	2.44	3.46
	Percent	100[a]	58	157	150	108[a]	60[a]	56[a]	72[a]	161[a]	168[a]
III	Log10	1.00	1.00	2.18	1.89	1.74	1.20	1.00	1.00	2.31	1.79
	Percent	100[a]	58	193	159	174[a]	120[a]	100[a]	100[a]	231[a]	179[a]
IV	Log10	1.00	1.00	1.40	0.65	1.00	0.78	1.30	0.91	2.11	2.65
	Percent	100[a]	60	157	132	141[a]	60[a]	72[a]	74[a]	161[a]	145[a]

[a] Superscripts which share a common letter within each treatment indicate that they were not significantly different ($p > 0.05$).
[b] Cheese type — See footnote to Table 3.
[c] Mean of counts ($\log_{10}$/g).
[d] Assuming mean count in milk to be 100% other counts expressed as the percentage of initial count.

Table 8–Fate of lactobacilli during manufacturing and storage of the four experimental types of Venezuelan queso blanco

Cheese type[d] (Treatment)		Stages of manufacture				Days (d) and temperature (°C) of storage					
		Milk	Whey	Curd	Salted curd	0 day	2 days 3°C	5 days 3°C	8 days 3°C	5 days 15°C	8 days 15°C
I	Log_{10}[e]	2.54	2.65	4.67	4.56	3.89	5.35	4.91	5.64	6.19	7.38
	Percent[f]	100^{a}	86	119	117	210^{ab}	264^{b}	311^{b}	321^{bc}	328^{bc}	390^{c}
II	Log_{10}	2.54	2.55	3.94	3.82	3.70	4.31	4.30	4.32	5.52	6.71
	Percent	100^{a}	85	113	110	145^{ab}	254^{b}	280^{b}	276^{b}	340^{bc}	392^{c}
III	Log_{10}	2.07	1.64	3.21	3.23	3.48	3.78	3.73	4.52	5.26	6.84
	Percent	100^{a}	88	36	181	202^{ab}	221^{ab}	204^{ab}	254^{b}	283^{bc}	396^{c}
IV	Log_{10}	2.02	2.12	3.44	3.08	3.45	3.57	4.12	3.96	4.33	6.24
	Percent	100^{a}	87	199	177	199^{ab}	206^{ab}	253^{b}	228^{b}	227^{b}	370^{c}

[a–c] Superscripts which share a common letter within each treatment indicate that they were not significantly different ($p > 0.05$).
[d] Cheese type — See footnote to Table 3.
[e] Mean of counts (log_{10}/g).
[f] Assuming mean count in milk to be 100% other counts expressed as the percentage of initial count.

the initial staphylococci population of the milk. The effect of initial microbial load and its behavior during survival in cheese has been studied (Frank et al., 1977).

During manufacture, there was at least a one log cycle increase in total coliform numbers from the milk to curd (Table 6). For the four treatments, there was an average of a 25-fold increase, compared to an approximate ten-fold increase from normal concentration in the curd (Takahashi and Johns, 1959). The difference indicated a rapid growth of total coliforms in the 90-min period following salting of the curd. Storage of the cheese revealed little growth following the pressing operation. The general increase in total coliforms from the milk to 0 day cheese was significant ($p < 0.05$) only for low salt, low moisture cheeses. This could be attributed to the salt effect and longer time period for making this type of cheese (195 min compared to 110 min for the high moisture cheeses). No statistically significant increase in total coliforms was observed ($p > 0.05$), when the cheese types were stored at 3°C for 0 to 8 days. However, there was a significant change ($p < 0.05$) during 8 days storage at 15°C for both low and high moisture, low salt cheese.

The behavior of fecal coliforms (Table 7) during manufacture was almost the same as that for total coliforms. An increase in fecal coliform numbers during storage of the cheese was observed. However, the manufacturing conditions (type cheese) did not affect significantly ($p > 0.05$) the growth of these organisms. This can be attributed to the wide variation in viable counts.

During the manufacture period, an increase in the number of lactobacilli was observed (Table 8), mainly due to the concentration effect (10-fold). The number of lactobacilli decreased slightly in the salted curd, maintaining almost the same number as in the fresh cheese. During storage, there was a significant ($p < 0.05$) increase at 15°C (Table 8). The manufacturing variations (type cheese) did not significantly ($p > 0.05$) affect the growth of lactobacilli (Table 8).

Growth of native lactobacilli was rapid during the early stages of manufacture. However, growth did not result in sufficient acid production to lower the pH to a level that would prevent the growth of undesirable microorganisms. Additionally, the lactobacilli isolated were not common "starter culture" types but were an adventitious microflora as suggested by Ayres et al. (1980).

The pH of fresh queso blanco or the stored cheese suggests a product highly susceptible to microbial growth. Obviously, without any benefits of an inherent low pH, the shelf stability of this product is highly dependent on the temperature of storage. The results confirm that the temperature is the most important control factor during storage.

Conclusions based on observing the fate of the native microflora may be misleading. It could be argued that the wide variation in starting populations, biotypes, strains, and even recontamination during the process would make it difficult to study the manufacturing variables. This point was acknowledged and dealt with by using pure cultures; the results will be presented in future work. However, the point to be stressed here is that there is nothing in the normal manufacturing procedure for Venezuelan queso blanco to ensure its microbiological safety.

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Electrical Stimulation of Mutton

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ABSTRACT

Electrically stimulated ovine muscles, restrained from shortening during rapid chilling at 0-1 or 15-16°C, had lower Warner-Bratzler (WB) shear force values after 1 and 2 days aging at 0-1°C than unstimulated controls, but were not significantly different at ⩾4 days aging. Direct measurement of muscle fiber length showed that contraction values obtained for muscles assigned to go into rigor at 0, 15, 30 or 40°C were significantly less for stimulated muscles than for control muscles at 0°C, but of same magnitude or at rigor temperatures ⩾15°C. WB shear force values indicated that, at temperatures ⩾15°C, increase in tenderness due to stimulation became small after 7 days aging at 0-1°C, whereas at 0°C aging further increased improvement due to stimulation. Results were thus consistent with electrical stimulation reducing myofibrillar shortening at rigor temperature <15°C but at temperature ⩾15°C stimulation had the same effect as a few days aging.

INTRODUCTION

THE TENDERIZING EFFECT of electrical stimulation has been attributed to a variety of causes including (a) the reduction or avoidance of the effects of cold shortening (Chrystall and Hagyard, 1976; Davey et al., 1976), (b) an acceleration of the aging process (Savell et al., 1978, 1981; George et al., 1980; Elgasim et al., 1981), (c) increased activity of acid proteases (Dutson et al., 1980), (d) physical disruption of the myofibrillar structure (Savell et al., 1978; Will et al., 1980) and (e) alterations in the thermal stability of collagen (Judge et al., 1980).

Evidence for electrical stimulation reducing or preventing cold shortening is equivocal; some authors (Smith et al., 1977, 1979; Bouton et al., 1978, 1980; George et al., 1980; Whiting et al., 1981; Salm et al., 1983) have found evidence in sarcomere length data to support it, while others (Demeyer et al., 1980; Salm et al., 1981; Savell et al., 1977, 1979; Elgasim et al., 1981; Smith et al., 1977) have found no significant difference in sarcomere lengths between electrically stimulated and unstimulated meat. Much of the work was, however, carried out using beef under chilling conditions where cold shortening was unlikely (e.g. Elgasim et al., 1981) and it was more likely that muscle samples were going into rigor at temperatures ⩾15°C. Comparatively recent work (Locker and Daines, 1975, 1976) indicated that, for beef sternomandibularis muscle, muscle going into rigor shortened almost as much at 37°C as at 2°C, but had shear values of the same order of magnitude as those obtained for samples conditioned at 15°C. There was thus the possibility that, if stimulated muscles were going into rigor at temperatures greater than 30°C, then shortening would still occur without the concomitant toughening which would normally occur at temperatures near 0°C.

In this present paper the effect of allowing stimulated and unstimulated muscles to go into rigor at a wide range of temperatures (0–42°C) was investigated.

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MATERIALS & METHODS

Electrical stimulation

Electrical stimulation of the dressed sheep carcasses (all full mouth wethers – at least 4 yr old) was carried out with the carcass suspended from the Achilles tendon via an insulated hook. The live electrode was inserted in the neck while the earth electrodes were inserted in both back legs. Stimulation was carried out for 2 min within 20 min of slaughter. The RMS voltage was 800V with a peak voltage of 1140V at a frequency of 14.3 Hz. In experiments 2, 3 and 4 the gracilis muscles were, prior to stimulation, carefully peeled back to enable pins to be inserted into the then exposed semimembranosus (SM) muscles, so that length measurements of that muscle could be made before and after removal from the carcass. In experiments 5 and 6 the longissimus dorsi (LD) and semimembranosus (SM) muscles were removed from one side of each carcass prior to stimulation (to serve as unstimulated controls) – the carcass was then stimulated with these muscles missing. Deep butt temperatures were measured before and after electrical stimulation using a thermistor probe (Ebro-therm T181).

Conditioning, aging and cooking

Samples removed at 0.5 or 2 hr post slaughter for conditioning at 0, 15, 30, 35, 37, 40 or 42°C, were very loosely wrapped in polyethylene bags and totally immersed in either ice slush, at 0°C, or in water baths controlled at the selected temperature (±0.5°C). The conditioning times were 24 hr at 0 and 15°C. For the other temperatures the times used were 10 hr at 30 and 35°C, 7 hr at 37°C, and 6 hr at 40° and 42°C followed by the remainder of the time to 24 hr at 0°C. These times were selected as being sufficient to complete rigor mortis (Locker and Daines, 1975, 1976).

Carcasses used in one experiment were conditioned in chillers at 0-1°C or 15-16°C. These carcasses were hung via the pelvis and were placed in the chillers approximately 90 min post slaughter and after removal of the fat layer over the LD muscles. Temperature measurements indicated that the center muscle temperature of the LD and deep butt was <1°C in less than 6 hr and 9 hr, respectively, in the 0-1°C chiller and, correspondingly, <20°C within 2 hr and 5 hr in the 15-16°C chiller.

Where muscle samples were aged, they were vacuum sealed in gas impermeable bags and stored at 0-1°C for the selected period.

Samples were cooked in polyethylene bags totally immersed in water baths, temperature controlled at 80 ± 0.5°C for 60 min. Samples weighed between 50 and 80g.

Measurement of pH

The pH of muscles was measured using a probe type combined electrode (Phillips C64/1) with a portable pH meter (Watson Victor Model 5004). The pH was measured just prior to cooking but measurements were also made, when necessary, just before samples were placed in the chiller or immediately after removal of the muscle from the carcass, in order to determine the effect of electrical stimulation.

Experimental design

Experiment 1. Twenty sheep were assigned, 5 per group, to 4 treatments, i.e. electrical stimulation or no stimulation followed by conditioning at 0-1°C or 15-16°C. After slaughtering, dressing and stimulation (where required) all the carcasses were hung from the pelvis within about 20 min after slaughter. Approximately 90 min after slaughter (during which time the carcasses were at room temperature i.e. 20-25°C) the deep pectoral (DP) muscles were removed from each carcass and the carcass was then stored in the appropriate chiller. The LD, biceps femoris (BF) and SM muscles

were removed from the carcasses after 24 hr storage. Each pair of LD muscles provided 6 samples which were assigned, at random, to aging at 0-1°C for 1, 2, 4, 8, 16 or 32 days. Each pair of BF or SM muscles provided 4 samples which were similarly assigned to aging at 0-1°C for 1, 2, 4 or 8 days. After completion of each aging period the samples were cooked (at 80°C for 60 min), stored overnight at 0-1°C then prepared for Warner-Bratzler (WB) shear force measurements.

Each pair of DP muscles yielded 4 samples which were assigned to conditioning at 0°C and for 24 hr, 15° or 37°C for 7 hr, and 0°C for 24 hr plus 7 hr at 37°C (Locker and Daines, 1975, 1976). Each sample had pins placed at a known distance apart so that contraction along the muscle fiber length, produced by the various conditioning treatments, could be measured. These samples were cooked and prepared for WB shear force measurements as described previously.

Experiment 2. Twenty sheep were assigned to 2 treatments (10 per treatment), i.e. electrically stimulated or unstimulated (control). The pairs of LD and SM muscles were removed immediately after stimulation and/or about 30 min after slaughter from each carcass. Each LD muscle was split into 2 across its length and each SM muscle was split lengthwise. The 4 sub-samples from each pair of muscles were then assigned to conditioning at 0, 15, 30 or 40°C (42°C for the LD muscle). Pins were placed in the SM muscles on the carcass (after exposing the surface by careful dissection of the gracilis muscle which normally overlies it) prior to stimulation and the distances between the pins measured with the carcass first in its normal (Achilles tendon hung) position and again when hung via the pelvis. Distances between the pins were measured after excision and the amount of shortening in the control and stimulated muscles determined. Length changes in the SM muscle due to conditioning were measured as described previously. Each LD muscle sub-sample was halved after conditioning and aged for 1 or 7 days at 0-1°C. After treatment the samples were cooked and prepared from WB shear force measurements as previously.

Experiment 3. This experiment used 20 sheep and was similar to Experiment 2 except that the muscles were removed at 2 hr after slaughter.

Experiment 4. Eight sheep were electrically stimulated and the SM muscles were removed from one side of each carcass immediately after stimulation (as in Experiment 2) and from the other side 2 hr afterwards (as in Experiment 3). After removal from the carcass each SM muscle was split lengthwise into 3 sub-samples of approximately equal size which were then assigned to conditioning at 0, 15, or 40°C for the times previously used. Length changes during stimulation and conditioning were measured as previously described.

Experiment 5. The LD and SM muscles were removed from one side of each of 16 sheep carcasses, within 10-15 min of slaughter, before stimulation, while the muscles from the other side were removed after stimulation. Each muscle (both control and stimulated) was then divided into 4 approximately equal pieces. The 4 pairs of control and stimulated sub-samples from each muscle (SM and LD) from each of 8 animals were assigned at random to conditioning in polyethylene bags in a water bath at 39°C for 0, 30, 60 or 90 min. The zero time was approx. 30 min after slaughter. The 4 pairs of control and stimulated LD and SM muscles from each of the remaining 8 carcasses were similarly assigned but this time at 35°C fo 0, 30, 60 or 120 min. After conditioning for the required time at 35 or 39°C the samples were transferred to another water bath at 15°C for 24 hr. After conditioning at 15°C the samples were cooked and prepared for WB shear force measurements as previously described.

Experiment 6. One LD and SM muscle was removed from one side of each of 8 carcass before stimulation and the remaining LD and SM muscles within 5 min of electrical stimulation. Each LD was cut into 5 approximately equal sized samples and assigned at random to conditioning at 15, 30, 35, 40 or 42°C. Each SM muscle was divided into 4 approximately equal sized samples and assigned at random to 15, 30, 35 or 40°C. A small quantity of oil or water at the same temperature as the water bath was added to each sample bag to ensure free contraction. After conditioning for the appropriate time the samples were cooked and prepared, as before, for WB shear force measurements.

Shear force measurements

The WB shear force device used has been described previously (Bouton et al., 1975). Initial yield force values were measured from the WB shear force deformation curves. They were taken as the point at which the samples first began to yield. Throughout the test only initial yield force values have been quoted or used.

Statistical methods

Analysis of variance was used to determine the treatment effects, appropriate standard errors and least significant differences (LSD's), at the $P < 0.05$ level, between treatment means.

RESULTS & DISCUSSION

Experiment 1

The WB shear force results obtained for the control samples of BF and SM muscles were significantly greater than those obtained for the stimulated muscle samples, at up to 4 days aging for the samples conditioned at 0°C, and up to 2 days for those conditioned at 15°C (Table 1). At 8 days there was no longer a significant effect due to stimulation. The results obtained for the LD muscle samples were similar, although the control samples conditioned at 0°C had relatively high values compared to those conditioned at 15°C (Table 2). After 8 days, differences due to stimulation and conditioning temperature were small.

In work with ovine muscle (Møller et al., 1983), which had been effectively restrained from cold shortening, the tenderizing effect of electrical stimulation was small and not significant. Other work (George et al., 1980) with a restrained bovine muscle showed a small, albeit significant, decrease in shear force values with electrical stimulation – which became nonsignificant at 14 days aging. This difference could be attributed to chilling rates which were fairly rapid for the former (deep butt temperature reached <1°C in 11-12 hr) and relatively slow for the latter (8 hr at 16°C before storage in still air at 1°C). The data reported in Tables 1 and 2 contrasted the effect of stimulation followed by rapid chilling (deep butt temperature <20°C in 5 hr) on the shear properties of stretched (Table 1) and partially restrained (Table 2) muscle. The effect of using slow chilling

Table 1–WB initial yield force results obtained for SM and BF muscles from mutton carcasses hung from the pelvis and held at either 0 or 15°C for 24 hr after either no electrical stimulation or stimulation. Samples were cooked at 80°C for 1 hr after aging for 1, 2, 4 or 8 days storage at 0-1°C

Muscle	Muscle treatment[a]	Days aged				LSD[b]	
		1	2	4	8	Aging	Treatment
SM	0C	3.90	3.56	2.77	2.24	0.14	0.27
	0S	3.32	2.85	2.25	2.00		
	15C	3.07	2.70	2.16	1.97		
	15S	2.29	2.39	2.18	1.95		
BF	0C	3.53	2.95	2.38	2.02	0.04	0.18
	0S	3.33	2.41	2.12	1.99		
	15C	3.30	2.50	2.02	1.89		
	15S	2.44	2.29	1.98	2.00		

[a] Conditioning temperature 0 and 15°C. C — Control (unstimulated); S — stimulated.
[b] Least significant difference at P<0.05.

without stimulation was about the same as obtained by stimulation and then fast chilling.

For the DP muscles removed from the carcasses at about 90 min post slaughter, electrical stimulation had significantly decreased shear force values at 0, 15 and 37°C (Table 3). Shear force values were significantly lower at 15° than at either 0 or 37°C for both stimulated and control samples. Holding the '0°C' samples for a further 7 hr at 37°C had no large tenderizing effect as has been suggested (Locker and Daines, 1975, 1976). The extent of contraction with temperature was less at 15°C than at 0° or 37°C. While there was no significant difference between the contraction of stimulated and control samples at 15°C the former contracted considerably less at 0°C and slightly less at 37°C. The samples subjected to further conditioning at 37°C, after initially conditioning at 0°C (i.e. 0/37 in Table 3), contracted similarly and had similar shear force values to those conditioned at 0°C only.

The average pH values obtained for the stimulated LD, ST and DP muscles were 6.05, 5.80 and 5.97, respectively, at 90 min post slaughter. The equivalent control muscles had values of 6.94, 6.50 and 6.74.

Experiment 2

The results obtained for the LD muscles conditioned at 0, 15, 30 and 42°C then aged for 1 or 7 days (from slaughter) at 0 - 1°C are shown in Fig. 1. Compared to controls, stimulated animals had significantly lower LD shear force values for the 1 day aged, and 0°, 15° and 30° conditioned samples, but values of the same magnitude for the 42°C treatment. Aging for 7 days significantly reduced shear force values of all the 0°C and 15°C samples and for the 30°C samples from the control animals only but had no effect on values obtained for the samples conditioned at 42°C. In agreement with earlier results (Experiment 1 – Tables 1 and 2), aging for 7 days reduced the effect of stimulation to nonsignificance for samples conditioned at 15° or above. Aging did, however, significantly reduce the shear force values obtained for the samples conditioned at 0°C and, in fact, enhanced the effect of electrical stimulation (Fig. 1).

Stimulation of the SM muscles significantly reduced shear force values obtained at 0, 15, 30 and 40°C with the largest reduction at 0°C, relative to unstimulated muscles (Fig. 2). The measurement of the contraction in length of the raw samples during the conditioning treatments showed no significant effect due to stimulation for temperatures of 15°C and above but a highly significant reduction in the amount of shortening at 0°C. No significant difference was found between lengths of SM muscles before and after removal from carcasses which had or had not been stimulated (Table 4).

The mean pH values obtained for the stimulated LD and SM muscles, measured at about 30 min post slaughter, were 6.26 and 6.07, respectively. The corresponding control samples had pH values of 6.87 and 6.78 at this time.

Experiment 3

An attempt was made to simulate conditions which could pertain to muscles removed in a commercial-type hot boning operation; i.e. the muscles were removed at about 2

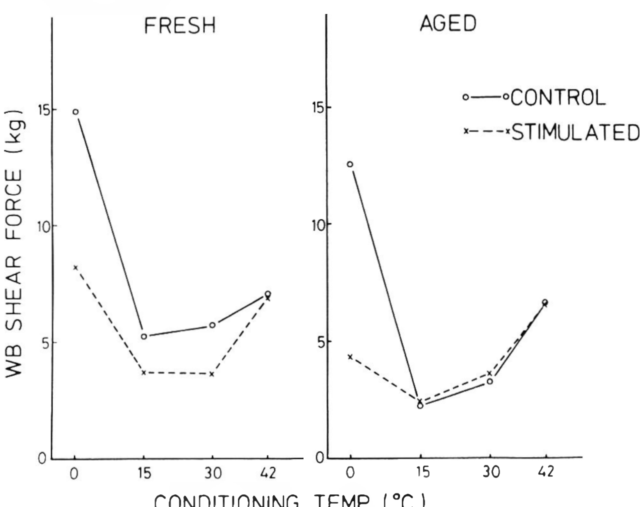

Fig. 1–Effect of aging for 1 or 7 days at 0 - 10°C and electrical stimulation on the WB shear force properties of ovine LD muscle removed immediately after stimulation and held at 0 and 15°C for 24 hr, 30°C for 10 hr and 42°C for 6 hr before cooking at 80°C for 1 hr. Standard error of means is 0.31 kg.

Table 2–WB initial yield force results obtained for LD muscles from mutton carcasses hung from the pelvis and held at either 0 or 15°C for 24 hr after either no electrical stimulation or stimulation. Samples were cooked at 80°C for 1 hr after aging for 1, 2, 4, 8, 16 or 32 days storage at 0-1°C

Muscle treatment[a]	Days aged						LSD[b]	
	1	2	4	8	16	32	Aging	Treatment
0C	7.91	4.60	4.03	2.94	2.16	2.75	0.57	1.22
0S	5.44	3.55	2.42	2.42	2.76	2.93		
15C	4.36	3.34	2.88	2.54	2.07	2.20		
15S	2.66	3.01	2.25	2.09	3.07	3.57		

[a] Conditioning temperatures 0 and 15°C. C — Control (unstimulated) S — Stimulated.
[b] Least significant difference at $P<0.05$.

Table 3–WB initial yield force values and length contraction obtained for DP muscles removed pre-rigor, from stimulated and unstimulated (control) mutton carcasses, and held at 0°C (24 hr), 15°C (24 hr), 37°C (7 hr) and 0°C (24 hr) + 37°C (7 hr). Samples were cooked at 80°C for 1 hr

Parameter measured	Treatment[a]	Conditioning temperature (°C)				LSD[b]	
		0	15	37	0/37	Temp.	Stim.
WB initial yield force (kg)	C	12.78	6.74	8.67	10.99	1.25	0.88
	S	6.12	4.18	5.75	4.24		
% Raw contraction	C	31.9	10.3	25.6	30.0	3.6	2.5
	S	11.3	8.8	20.6	8.1		

[a] C — Control (unstimulated); S — Stimulated.
[b] Least significant difference at $P<0.05$.

hr post slaughter. The results obtained for the LD (Fig. 3) and SM (Fig. 4) muscles when this treatment was used contrasted markedly with the results obtained for the LD (Fig. 1) and SM (Fig. 2) when muscles were removed (Exp. 2) soon after dressing and stimulation. The stimulated LD muscles conditioned at 15°C had higher shear values (albeit not significantly so for the fresh samples) than obtained for the unstimulated controls (Fig. 3). The difference was significant in the LD muscle sampled after 7 days aging. Stimulation significantly reduced shear force values for both SM and LD muscles conditioned at 0°C (Fig. 3 and 4) but was clearly ineffective for samples conditioned at the higher temperatures. The length contraction (%) values for the SM appeared to show that stimulation had reduced contraction during conditioning at 0, 30 and 40°C. Length measurements showed, however, that SM muscles excised from the carcass 2 hr after stimulation were significantly

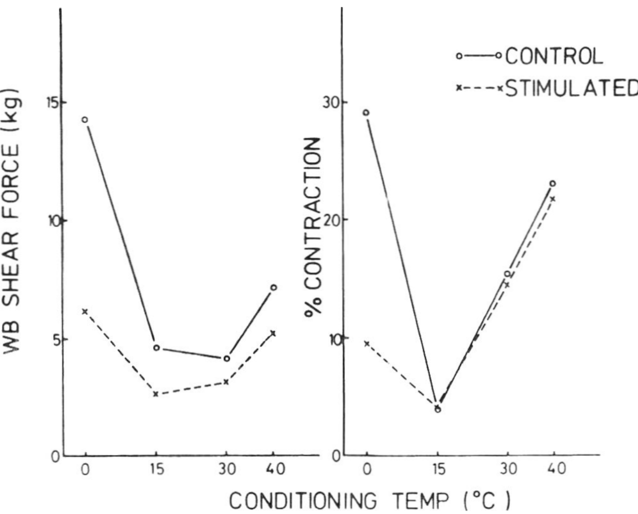

Fig. 2—Effect of electrical stimulation on the WB shear force and length (raw) contraction of ovine SM muscle removed immediately after stimulation and held at 0° and 15°C for 24 hr, 30°C for 10 hr and 40°C for 6 hr before cooking at 80°C for 1 hr. Standard error of means is 0.45 kg for WB shears and 2.1 for % length contraction.

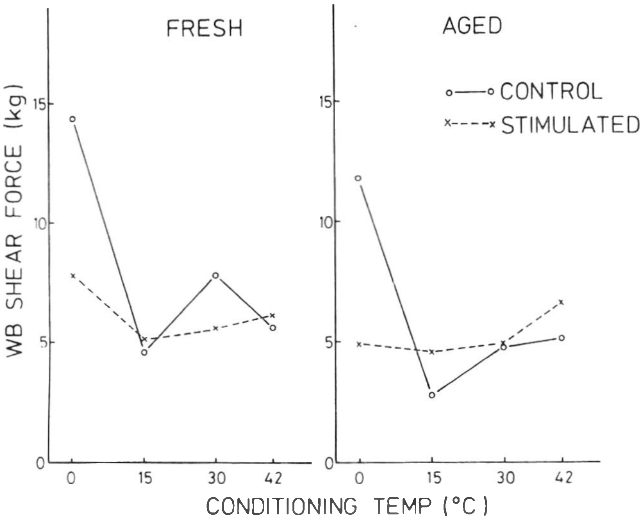

Fig. 3—Effect of aging at 0 - 1°C for 1 or 7 days and electrical stimulation on the WB shear force properties of ovine LD muscle removed 2 hr after slaughter and held at 0 and 15°C for 24 hr, 30°C for 10 hr and 42°C for 6 hr before cooking at 80°C for 1 hr. Standard error of means is 0.31 kg.

($P < 0.001$) shorter than the muscles from the control animals (Table 4) so that the stimulated muscles had shortened on the carcass prior to removal.

The average pH values obtained for the stimulated SM, LD and BF muscles measured at about 2 hr post slaughter were 5.73, 5.72, and 5.75 compared with the controls 6.52, 6.64, and 6.77, respectively, at the same time.

Experiment 4

The excised lengths of SM muscles removed immediately after stimulation (Treatment A) were compared with those removed 2 hr later (Treatment B). The results (Table 5) showed that the muscles removed at 2 hr (B) were appreciably and significantly shorter than those removed earlier from the carcass (A).

The shear force values obtained for the samples conditioned at 15°C were significantly higher for those muscles removed at 2 hr from the carcass. There was no significant difference, attributable to the time when the muscles were removed, for the samples conditioned at 0 or 40°C. The contraction of the raw samples during conditioning was markedly greater for those removed immediately. Initial pH values were well below 6.0 for both treatments although the samples from the B treatment had significantly lower values.

Experiment 5

Control (unstimulated) and stimulated LD and SM muscles were removed from carcasses within 30 min of

Table 4—Length contraction values obtained for sheep SM muscles excised from the carcasses either before or after electrical stimulation within either 30 min of slaughter (Exp. 2) or 2 hr of slaughter (Exp. 3)

Parameter measured	Exp. No.	Treatment Control	Stimulated	LSD[b]
L/L_{AT}	2	1.17	0.96	0.15
	3	1.08	1.05	0.10
L/L_{TS}	2	0.65	0.58	0.04
	3	0.64	0.60	0.05

[a] L — Excised length; L_{AT} — Length when carcass hung via the Achilles tendon; L_{TS} — Length when carcass hung via pelvis.
[b] Least significant difference at $P<0.05$.

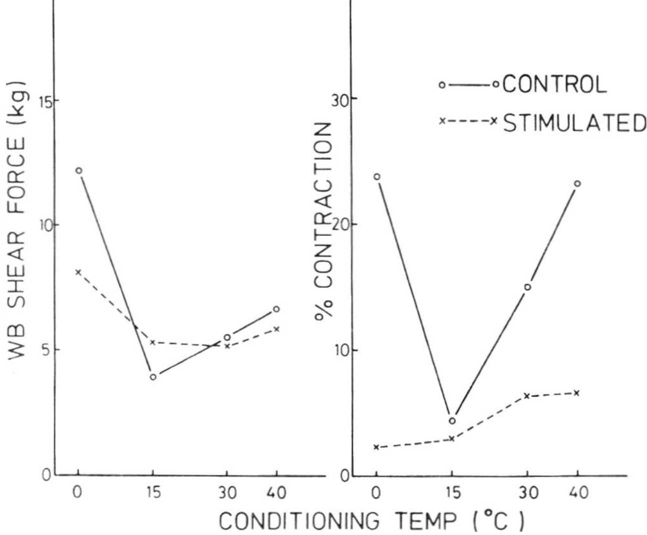

Fig. 4—Effect of electrical stimulation on the WB shear force and length (raw) contraction of ovine SM muscle removed 2 hr after slaughter and held at 0 and 15°C for 24 hr, 30°C for 10 hr and 40°C for 6 hr before cooking at 80°C for 1 hr. Standard error of means is 0.45 kg for WB shear and 1.7 for % length contraction.

slaughter and held at 39° or 35°C before they were transferred to 15°C. For the control LD muscles shear force values were not significantly affected by prior holding time at 39°C (Fig. 5). The shear force values obtained for the stimulated LD muscle samples, however, increased with holding time at 39°C. The SM muscles behaved a little differently since the shear force values obtained for the control muscles declined with time while the values obtained for the stimulated muscles increased with time at 39°C.

The differences between ultimate pH and the pH values, after removal from the 39°C environment, (i.e. ΔpH), of the control SM muscles were significantly less than those of the LD muscle at each sampling time (Fig. 6). The ΔpH values obtained for the stimulated muscles were very much lower than the equivalent values for the control muscles. Both stimulated muscles had effectively reached ultimate pH at 60 min postmortem. The ultimate pH values obtained for the control SM and LD muscles were 5.50 and 5.51, respectively, which were significantly ($P < 0.01$) less than corresponding stimulated muscles (both 5.58).

Table 5—WB initial yield force, raw contraction (%) during conditioning, initial pH, length contraction when the SM muscles were excised from the carcass immediately after electrical stimulation (A) and 2 hr after stimulation (B) before conditioning at 0°C (24 hr), 15°C (24 hr) and 40°C (6 hr)

Parameter measured	Treatment	Conditioning temp. (°C) 0	15	40	LSD[a]
WB initial yield force (kg)	A	6.62	4.34	9.10	1.44
	B	6.32	5.94	8.86	
% Raw contraction	A	16.7	15.7	25.0	4.6
	B	6.7	5.5	4.9	
Initial pH	A	5.88	–	–	0.04
	B	5.75	–	–	
L/L_{AT}[b]	A	1.16	–	–	0.13
	B	0.99	–	–	
L/L_{TS}[c]	A	0.64	–	–	0.06
	B	0.56	–	–	

[a] Least significant difference at $P<0.05$.
[b] L — Excised length; L_{AT} — Length when carcass hung via Achilles tendon.
[c] L_{TS} — Length when carcass hung via pelvis.

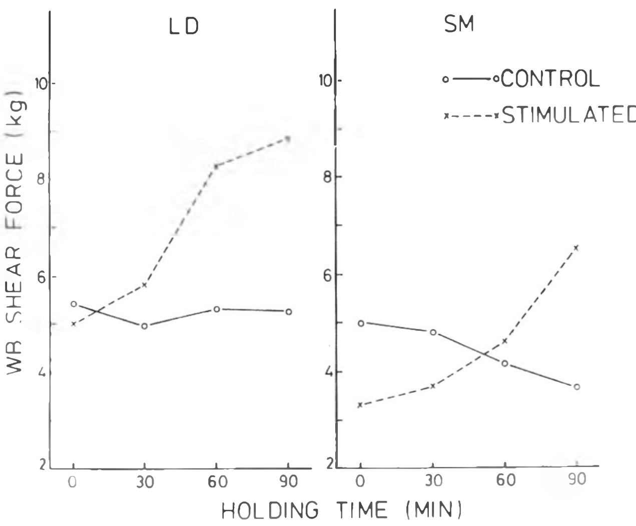

Fig. 5—WB shear force values obtained for LD and SM muscles removed from sheep carcasses either within a few minutes of electrical stimulation or just before stimulation and held at 39°C for 0, 30, 60 or 90 min before transferring to 15°C for 24 hr then cooking at 80°C for 1 hr. Standard error of means is 0.41 kg for the LD and 0.32 kg for the SM muscles.

The shear force results for the stimulated and control LD and SM muscles held at 35°C are similar to those obtained for the muscles held at 39°C (Fig. 7). At this 35°C holding temperature the shear force values obtained for the control muscle samples decreased with sample holding time while shear force values obtained for the stimulated samples increased. The ΔpH values (Fig. 8) obtained for the SM muscles were again lower than those obtained for the LD muscles (see ΔpH values in Fig. 6) for both the control and stimulated samples. The ultimate pH values obtained for the control SM (5.48) and LD (5.50) were significantly ($P < 0.01$) less than the corresponding stimulated muscles (5.53 and 5.55 respectively).

Measurements of length changes for both the samples held at 39 and 35°C were more variable than in earlier experiments due to the small sample sizes. At 39°C, however, the stimulated samples contracted significantly ($P < 0.05$) more during conditioning than the controls (16.8% vs 9.6%) and the contraction of the stimulated samples was significantly ($P < 0.05$) greater between 0 and

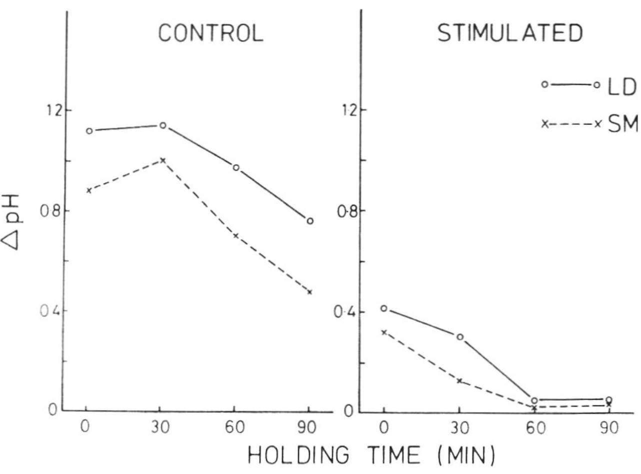

Fig. 6—ΔpH (initial-ultimate pH) values obtained for stimulated and unstimulated LD and SM muscles removed from carcass just before or just after stimulation and measured after holding at 39°C for 0, 30, 60 or 90 min. Standard error of means is 0.03 for the LD and 0.04 for the SM muscles.

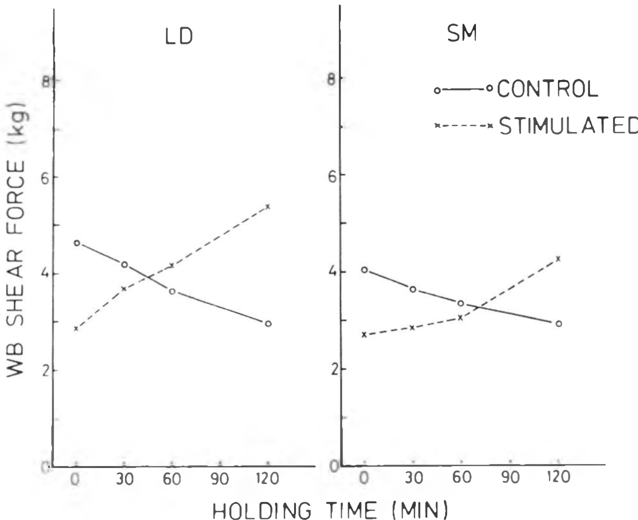

Fig. 7—WB shear force values obtained for stimulated and unstimulated LD and SM muscles removed from carcass either before or soon after stimulation, held at 35°C for 0, 30, 60 or 120 min before holding at 15°C for 24 hr then cooking (for shear) at 80°C for 1 hr. Standard error of means is 0.30 kg for the LD and 0.32 kg for the SM.

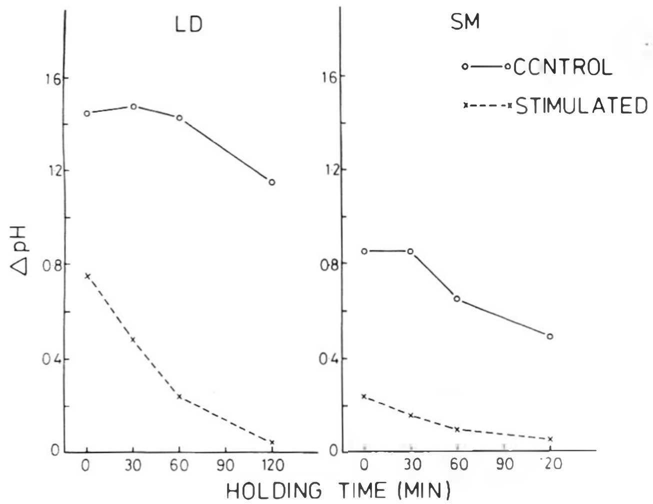

Fig. 8—ΔpH (initial-ultimate pH) values obtained for stimulated and unstimulated LD and SM muscles removed from carcass, either before or soon after stimulation, and held at 35°C for 0, 30, 60 or 120 min before holding at 15°C for 24 hr. Initial pH was measured at the end of the holding period at 35°C. Standard error of means is 0.06 for the LD and 0.05 for the SM muscles.

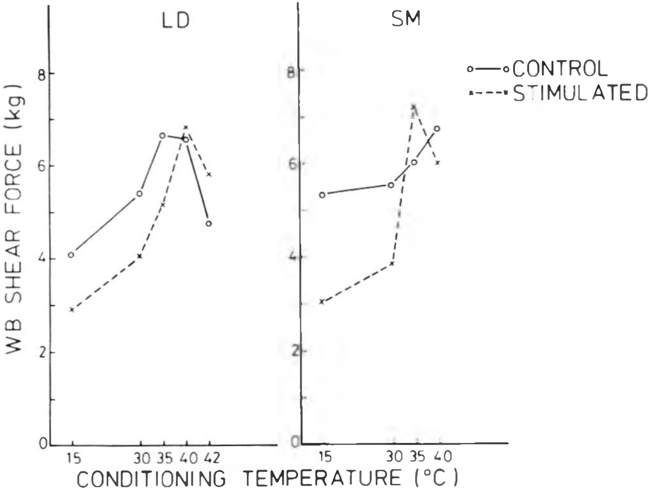

Fig. 9—WB shear force values obtained for LD and SM muscles removed either after stimulation or before stimulation then held at 15, 30, 35, 40 or 42°C (LD) or 15, 30, 35 or 40°C for the SM muscle. Holding times were 24 hr for 15°C, 10 hr for 30 and 35°C and 6 hr for 40 and 42°C. Samples were cooked at 80°C for 1 hr. Standard error of means is 0.39 kg for the LD and 0.59 kg for the SM muscles.

90 min holding times (9.6 - 22.8%). For the 35°C samples the stimulated samples contracted more than the controls (12.7% vs 8.7%) but the difference was not significant. There was an increase in contraction (9.4 vs 14.4%) with holding time for the stimulated samples but the difference was not significant.

Experiment 6

The WB shear force results obtained for the control and stimulated LD and SM muscles conditioned at temperatures between 15 and 42°C indicated (Fig. 9) a steep increase with rigor temperature in shear force values for both control and stimulated muscles. Muscles going into rigor at temperatures well above 30°C could show a poor reaction to electrical stimulation.

Average deep butt temperatures of the sheep before stimulation, 39.4°C, rose to 41.2°C after it. In a few cases the increase was considerably greater than 1.8°C. It was evident, however, that stimulation significantly ($P < 0.001$) increased deep butt temperatures.

CONCLUSIONS

IT HAS BEEN SHOWN (Tables 1 and 2) that electrical stimulation significantly reduced shear force values for sheep carcasses subjected to either fast or slow chilling but after 4 days aging for the former and 2 days for the latter the effect of stimulation was no longer significant. These results thus indicated that if myofibrillar shortening was avoided or minimized by restraint or by conditioning at 15°C then the effect of stimulation was transient. Stimulation thus appeared to produce a decrease in shear force values which after a few days aging disappeared. It could, therefore, be said to accelerate aging.

The results obtained for the DP muscles (Table 3) contrasted the effect of using different conditioning temperatures viz. 0, 15 and 37°C. Stimulation significantly reduced shear values at all 3 temperatures. The changes in muscle fiber length during conditioning indicated that the greatest effect of stimulation occurred at 0°C and that there was considerable contraction at 37°C for both control and stimulated samples. It has been suggested (Locker and Daines, 1975, 1976) that the high shear values obtained for samples cold shortened by being held at 0°C for 24 hr could be substantially reduced by holding for a further 7 hr at 37°C. In Table 3 it can be seen that holding at 37°C significantly reduced shear force values but not to the level obtained by conditioning at 15°C.

The results obtained for muscles removed within 30 min of slaughter and conditioned at different temperatures (Fig. 1 and 2) showed that aging for 7 days at 0 - 1°C virtually eliminated the differences due to stimulation which existed for the unaged samples conditioned at 15 or 30°C but for those samples conditioned at 0°C aging accentuated the difference due to stimulation. This result indicates that stimulation and aging had virtually the same effect for samples conditioned at 15°C or higher while at 0°C stimulation has reduced myofibrillar shortening. Support for this suggestion came from the direct measurements of length changes during conditioning (Table 3) since it was shown that, at temperatures of 15°C or more, stimulated and unstimulated samples contracted by similar amounts. At temperatures below 15°C where cold shortening could occur stimulation greatly reduced contraction.

When muscles were removed from the carcass about 2 hr after stimulation the shear force and length contraction results (Fig. 3 and 4) were markedly different from those obtained when the muscles were removed very soon after stimulation (Fig. 1 and 2). Delay in removing the muscles from the carcass apparently meant that the stimulated muscles contracted far less than the controls. It also seemed that stimulation actually increased the shear force values obtained for samples conditioned at 15°C. Other results, however, (Table 4 and 5) showed that the stimulated muscles had already shortened on the carcass prior to removal.

Stimulated muscle held at relatively high temperatures (35 or 39°C) for more than about 60 min before cooling to 15°C had higher shear values than similarly treated controls (Fig. 7 and 9). It thus appeared that on a carcass, under normal chilling conditions, stimulated muscles could go into rigor at temperatures higher than unstimulated control muscles and, in consequence, undergo greater shortening and have higher shear force values. For stressed animals and others which can go into rigor at relatively high temperatures shear values could be high.

In the 'Introduction' it was pointed out that the evidence for electrical stimulation preventing cold shortening was equivocal. The evidence put forward in this paper has indicated (a) that if chilling rates were fast enough to produce cold shortening in unstimulated samples free to shor-

ten then stimulation reduced the amount of cold shortening and (b) that, if chilling rates were such that stimulated and unstimulated samples went into rigor at temperatures above 15°C, stimulation had little influence on myofibrillar shortening and the main effect was to accelerate aging – an effect which disappeared after a few days aging.

Since stimulated samples shortened nearly as much as unstimulated samples when both went into rigor at the same temperature (for temperatures > 15°C) it appeared that under normal chilling conditions stimulated muscles could go into rigor at higher temperatures than unstimulated controls. The rate at which muscles go into rigor could depend on factors other than stimulation or temperature such as stress. Muscles from stressed animals after stimulation could go into rigor at temperatures considerably higher than without stimulation with resulting higher shear force values and toughness.

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CRABMEAT PASTEURIZATION . . . From page 1004

and food technologists working in the marine area. The statement is true for work with thermal death time tubes: however, in the "standard" process of heating 401 x 301 cans containing 454g of crabmeat to a temperature of 85°C and holding 1 min before cooling results in a process that is more than adequate since there is significant lethality in heating and cooling of the product. Our data suggest that $F_{185}^{16} = 31$ could be considered for use by the pasteurized crabmeat industry as the standard process, irrespective of can size or container type.

Table 1—Heating and cooling parameters for the various containers of crabmeat pasteurized to 85°C (185°F) for 1 min

Weight of meat (g)	Size of containers	f_h	j_h	f_c	j_c
454.0	pouch[a]	51.5	1.4	56.7	1.1
454.0	303 x 406	55.5	1.5	75.6	1.4
454.0	401 x 301	62.0	1.5	93.0	1.4
227.0	pouch[b]	25.5	1.5	27.6	1.0
227.0	307 x 206	42.3	1.5	59.0	1.2
113.5	pouch[c]	16.7	1.5	24.6	1.0
113.5	211 x 114	24.0	1.6	34.5	1.2

[a] 17.8 x 14.6 x 4.5 cm
[b] 12.7 x 12.6 x 3.4 cm
[c] 12.7 x 11.4 x 2.6 cm

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An Enzyme Immunoassay Technique for Detection of Salmonellae in Meat and Poultry Products

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ABSTRACT

A commercially available enzyme immunoassay (ELISA) in which a myeloma protein (MOPC 467) is used for detection of salmonellae was compared with two conventional cultural methods for detection of salmonellae in naturally contaminated meat and poultry products. Products tested included mechanically deboned poultry, chitterlings, poultry carcass rinsings, chicken necks, luncheon loaf emulsion, pork sausage and basturma. There was 100% agreement between ELISA and cultural methods. The ELISA technique is specific and rapid. Identification of *Salmonella*-contaminated meat and poultry products was accomplished in 2-3 days compared to the 4-6 days required by conventional cultural methods.

INTRODUCTION

THERE IS a pressing need in the food industry for an accelerated procedure which will detect the presence of salmonellae in a product. When present in foods these bacteria often are sublethally injured and nearly always are low in number in comparison with the total bacterial flora. Conventional methods of analysis of foods for salmonellae involve the use of one or more selective broths, usually preceded by nonselective enrichment; selective enrichment broths have to be streaked onto plates of differential medium. By the time the plates have been incubated and examined, and provided salmonellae are not found, 72 hr have elapsed in the attempt to declare the sample salmonella-free. On the other hand, if colonies resembling salmonellae are seen on the plates, the use of selective media and various biochemical and serological tests add from 24-72 additional hr to the analysis. The total time required to complete analysis of a salmonella-positive sample depends on whether it is considered necessary fully to serotype a strain that has been isolated. Often the industry need is not so much actually to isolate strains of salmonellae as it is to know whether or not the organisms are present in the cultured product.

Antigen-antibody reactions offer a means of recognition of the presence of salmonellae in a broth culture; fluorescent antibody tests have been in use for a number of years and more recently attention has turned to enzyme immunoassays (ELISA, EIA). Krysinski and Heimsch (1977) used enzyme-labeled antibodies to detect salmonellae on membrane filters. Minnich et al. (1982) developed an ELISA method which used immunoglobulin G(IgG) concentrated from commercial *Salmonella* polyvalent-H antiserum by removal of the IgM content. IgM is the primary immunoglobulin class elicited by somatic antigens. However, they reported that higher titer antiflagella antibody would be helpful. Two other *Salmonella* enzyme immunoassay methods, both of which use commercial polyvalent-H antiserum, have recently been reported (Aleixo et al., 1983; Anderson and Hartman, 1983).

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Robison et al. (1983) developed an ELISA procedure which uses antibody produced in mice by the mineral oil-induced plasmacytoma (MOPC 467) of Potter (1970). This antibody appears to bind to an antigen that is universal, or nearly so, among the salmonellae and is regarded as a flagella-related antigen (Potter, 1971; Smith et al., 1979; Smith and Potter, 1975). The procedure has been refined by Mattingly and Gehle (1984) and is now available as the *Salmonella* Bio-Enzabead Test Kit (Litton Bionetics, Inc., Laboratory Products Division, Charleston, SC 29405).

The present study was undertaken to evaluate this Kit and to compare results obtained by cultural procedures used in our laboratory for detection of salmonellae in meat and poultry products with the ELISA results.

MATERIALS & METHODS

Samples

All samples except the poultry carcass rinsings were divided into subsamples of 25g each. A sample of basturma, a fermented meat product that was known to be contaminated with salmonellae, was obtained from an FSIS field service laboratory; it was divided into two subsamples. The other material analyzed was selected without knowledge of whether it contained salmonellae. Five separate lots of chitterlings were each divided into two subsamples. A raw luncheon loaf emulsion containing pork was divided into 12 subsamples. One lot of mechanically deboned poultry was divided into 10 subsamples. One lot of chicken necks (skin included) was divided into 12 subsamples. Three different brands of fresh pork sausage obtained at a retail store were each subdivided into four subsamples. Six poultry carcass rinsings in lactose broth (about 80g each) were collected at a poultry processing plant. All samples were kept frozen until time of analysis.

Cultural methods

Half the subsamples, except for poultry carcass rinsings, were placed directly into TT broth, the tetrathionate medium of Hajna and Damon (1956); the rest were nonselectively enriched in lactose broth (Fig. 1). The procedures were those described in the "Microbiology Laboratory Guidebook" (USDA, 1974), with the following exceptions: (1) 0.2 mL instead of 0.5 mL of the lactose broth pre-enrichment culture was transferred to 10 mL TT broth; (2) TT broth was incubated at 42-43°C instead of 35°C; and (3) double-modified lysine iron agar (DMLIA) was used in addition to BGS and XLD agars as selective differential plating media. DMLIA was prepared as described by Rappold and Bolderdijk (1979), with the addition of 6.76 g/L sodium thiosulfate and 0.3 g/L ferric ammonium citrate (Chiu et al., 1983).

Antigen preparation

Antigens for ELISA were prepared at three different stages of the conventional cultural process: (1) lactose broth nonselective enrichment; (2) TT broth selective enrichment; and (3) TT broth direct enrichment. One-tenth milliliter of each of the enrichment broths was transferred to separate tubes containing 10 mL of M broth (Difco). M broth was devised by Sperber and Deibel (1969) to enhance flagellar production by bacteria. All M broth cultures were incubated at 37°C for 6 hr before antigen extraction. The bacteria were harvested from the M broth cultures by centrifugation at 1000 x *g* for 20 min. The cell pellets were resuspended in 2 mL phosphate buffered saline (PBS, pH 7.4) and heated for 1 hr (20 min is sufficient) in a boiling water bath. The heat-treated cell suspensions thus obtained were the antigens used in the ELISA test and were stored at 4°C until needed.

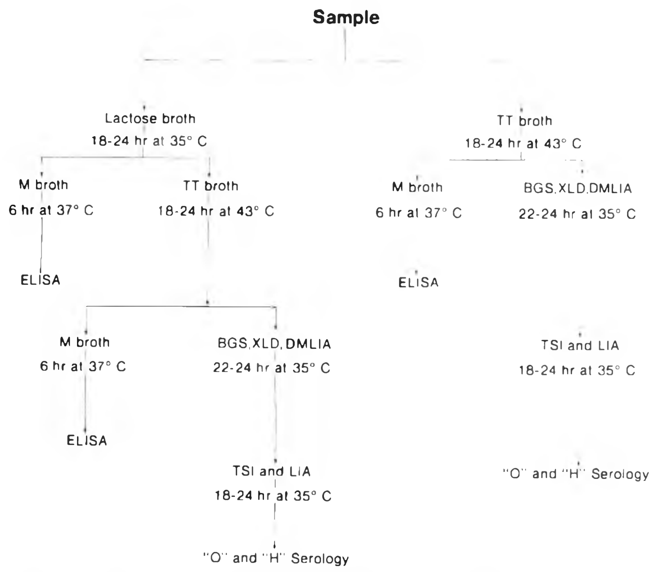

Fig. 1—Protocol used for detection of Salmonella *in naturally contaminated meat and poultry products by conventional cultural techniques and ELISA.*

ELISA procedure

The *Salmonella* Bio-Enzabead Test Kit makes use of the double antibody sandwich technique for the ELISA test by means of plastic-coated, ferromagnetic beads that have MOPC 467 antibody adsorbed to the plastic surface. The tests are performed in 96-well plastic flat-bottom microtitration plates. Antigen is added, in the wells, to 200 μL of PBS diluent containing 0.05% Tween-20, bovine serum and Thimerosal. Antibody-coated beads are placed in another 96-well plate. To start the assay, the beads are added to the diluted antigen preparations in the sample plate, using a Magnetic Transfer Device (Litton Bionetics, Inc.). The plates are incubated at 37°C for 20 min with gentle agitation after which the Magnetic Transfer Device is used to lift the beads and release them into another plate where they are washed in 0.05% Tween-20 wash solution (300 μL/well). The wash procedure is performed by raising and lowering the beads in the wash solution 12 times. The washed beads are raised once more with the Magnetic Transfer Device and transferred to another plate which contains horseradish peroxidase-labeled MOPC 467 antibody conjugate (200 μL per well). The plate is incubated at 37°C for 20 min with gentle agitation to permit attachment of conjugate to antigen. The beads are then washed as before and transferred to a plate containing 200 μL/well of 0.03% ABTS(2,2'-Azino-di[3-ethyl-benzthiazoline-sulfonate]) substrate dissolved in hydrogen peroxide solution. In the presence of horseradish peroxidase and hydrogen peroxide, ABTS is oxidized with development of an emerald green color. After a 5- or 10-min incubation period (without agitation) at room temperature, the enzyme reaction is stopped by addition of 25 μL of 1.25% sodium fluoride solution to each well. The beads are removed from the plate with the Magnetic Transfer Device, and the test results can be read visually against a white background or quantitated by measuring absorbance at 405-415 nm on an ELISA microplate reader.

In these experiments we put 25 or 100 μL of antigen preparation into the diluent and conducted the test by the Kit procedure described above. Positive wells were emerald green in color; negative wells were colorless. The intensity of color from negative (–) to 4+ was recorded. Known positive and negative control cultures were tested in each 96-well plate.

RESULTS & DISCUSSION

RESULTS OBTAINED by ELISA agreed 100% with the lactose broth/TT broth cultural method (Table 1). All 16 subsamples which were *Salmonella*-positive by the cultural method were also positive by ELISA; there were no false-positive ELISA results. ELISA results were obtained in 3 days; cultural method results of positive subsamples in 5 days, negative subsamples in 4 days.

When samples for ELISA testing were taken one step earlier in the culturing procedure from the lactose broth cultures, only 50% (8/16) of the subsamples which were positive by the cultural method were also positive by ELISA (Table 1). It is estimated that there must be a concentration of 10^6 salmonellae/mL of M broth culture in order for a sample to be positive by the ELISA method (Robison et al., 1983), and growth in lactose broth at 35°C overnight followed by a 6-hr incubation at 37°C in M broth apparently did not result in sufficient numbers of salmonellae for ELISA detection. This was not a surprising result for nonselective growth in the presence of competing microorganisms. The ELISA results were obtained in 2 days, but only half of the *Salmonella*-positive subsamples were detected; subsamples negative by the cultural method were also negative by ELISA.

Table 2 provides a comparison of ELISA results with those obtained by the TT broth direct enrichment cultural method. There was 100% agreement between the two methods. All 14 meat and poultry subsamples which were *Salmonella*-positive by the cultural method were also positive by ELISA. The ELISA results were obtained in 2 days;

Table 1—Comparison of the ELISA with the lactose broth preenrichment/TT broth selective enrichment cultural method for Salmonella detection

Product	Subsample no.	Cultural method Lactose/TT	ELISA Lactose/TT/M broth	ELISA Lactose/M broth
MDP[a]	1	+(B)[b]	4+[c]	3+[c](4+)[d]
	2	+(B)	3+	3+(4+)
	3	+(B)	3+	–(3+)
	4	+(B)	2+	1+(4+)
	5	+(B)	2+	1+(4+)
Chitterlings	6	+(B)	4+	–(2+)
	7	–	–	–(–)
	8	+(B)	4+	–(–)
	9	–	–	–(–)
	10	–	–	–(–)
Poultry carcass rinsings	11	–	–	–(–)
	12	–	–	–(–)
	13	–	–	–(–)
	14	–	–	–(–)
	15	–	–	–(–)
	16	–	–	–(–)
Chicken necks	17	+(B)	4+	–(–)
	18	+(B)	4+	–(1+)
	19	–	–	–(–)
	20	+(B)	4+	–(–)
	21	+(B)	4+	–(1+)
	22	–	–	–(–)
Luncheon loaf	23	–	–	–(–)
	24	–	–	–(–)
	25	–	–	–(–)
	26	–	–	–(–)
	27	–	–	–(–)
	28	–	–	–(–)
Pork Sausage A	29	+(B)	4+	–(–)
	30	+(B)	4+	–(–)
Pork Sausage B	31	+(C_1,C_2)	4+	–(–)
	32	+(C_2)	4+	–(–)
Pork Sausage C	33	–	–	–(–)
	34	–	–	–(–)
Basturma	35	+(E)	4+	–(–)

[a] MDP = Mechanically deboned poultry.
[b] Letters in parentheses represent O-Groups of the Salmonella isolates.
[c] 25 μL antigen; 5-min incubation in ABTS.
[d] 100 μL antigen; 10-min incubation in ABTS.

Table 2—Comparison of the ELISA with the TT broth direct enrichment cultural method for Salmonella detection

Product	Subsample no.	Cultural method TT Direct	ELISA TT/M broth
MDP[a]	36	+(B)[b]	4+[c]
	37	+(B)	1+
	38	+(B)	2+
	39	+(B)	1+
	40	+(B)	3+
Chitterlings	41	+(B)	4+
	42	–	–
	43	–	–
	44	–	–
	45	–	–
Chicken necks	46	–	–
	47	+(B)	2+
	48	–	–
	49	+(B)	4+
	50	–	–
	51	+(B)	2+
Luncheon loaf	52	–	–
	53	–	–
	54	–	–
	55	–	–
	56	–	–
	57	–	–
Pork sausage A	58	+(B,C_1)	4+
	59	+(B)	4+
Pork sausage B	60	+(C_2)	4+
	61	+(C_2)	4+
Pork sausage C	62	–	–
	63	–	–
Basturma	64	+(E)	4+

[a] MDP = mechanically deboned poultry.
[b] Letters in parentheses represent O-Groups of the Salmonella isolates.
[c] 25 μL antigen; 5-min incubation in ABTS.

cultural method results of positive subsamples in 4 days, negative subsamples in 3 days. Again, there were no false negatives.

The MOPC 467 antibody used in this ELISA method is unique in the broad spectrum of *Salmonella* serotypes it reacts with; it recognizes a large number of different serotypes. Of 100 strains tested by Robison et al. (1983), 94% were detectable with this antibody. No cross-reactivity with other enterics, such as *Escherichia coli,* has yet been found.

Because handling and storage of food products are costly to industry, application of this ELISA technique in laboratories monitoring food production should prove to be timesaving and therefore more economical. Sample analyses can be completed in 2-3 days compared to the 4-6 days currently required by cultural methods. Testing is necessary to assure the microbiological safety of products which could be subject to recall or seizure, and this quicker method of salmonellae detection would permit more rapid release of finished product and may even encourage increased monitoring of critical control points.

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Effects of Beef Carcass Electrical Stimulation and Hot Boning on Muscle Display Color of Polyvinylchloride Packaged Steaks

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ABSTRACT

Ninety-six beef sides from 48 carcasses were used to determine the effects of control (C, chilled 48 hr at 5°C), electrical stimulation (ES, 45 min postmortem, 400 volts for 2 min, pulsed), hot boning (HB, 2 hr postmortem), and combination (ESHB) treatments on muscle color of longissimus (LD) and semimembranosus (SM) steaks packaged in polyvinylchloride film. LD from HB was mostly visually darker, had less oxymyoglobin, and more metmyoglobin than other treatments as was the SM, but SM had fewer differences between HB and ESHB. ES and ESHB muscles were visually similar, suggesting ES minimized the darkening effect of HB. Regardless of treatment, muscle color was acceptable at 0, 1, 3 and 5 days of display.

INTRODUCTION

BESIDES LEANNESS (Jeremiah et al., 1972), the single most important merchandising characteristic of meat, particularly fresh beef, is meat color (Landrock and Wallace, 1955). If product is rejected because of color, the remaining sensory attributes may never be experienced.

Muscle visual color is largely due to relative proportions of oxymyoglobin, metmyoglobin, and reduced myoglobin. These are affected by packaging, length of display time (Pirko and Ayres, 1957; Pierson et al., 1970; Livingston and Brown, 1981), and processing. Electrical stimulation of carcasses, imposed early postmortem, has improved the color of beef and lamb muscles (Cross et al., 1979; Riley et al., 1980), with some exceptions (Grusby et al., 1976). ES carcasses at 24 hr had a brighter colored, firmer lean, and did not exhibit "heat ring" at 24 hr as compared to conventionally handled calf carcasses (Smith et al., 1977). ES on unsplit calf carcasses did not affect muscle color at 68 – 72 hr postmortem (Smith et al., 1979). Hall et al. (1980) found no differences between muscles from ES and non-ES carcasses in muscle color, surface discoloration, or overall appearance for ground beef up to 3 days of display. However, at 5 days of display ES round steaks were brighter and exhibited less surface discoloration than those from non-ES sides. Hall et al. (1980) proposed that the grinding and mixing process allowed for more complete oxygenation.

Kastner et al. (1973) and Henrickson et al. (1974) investigated the effects of hot boning (HB) on muscle color of beef carcass muscles excised after a 2, 3, 5 or 7 hr postmortem conditioning period at 16C, then stored in cryovac bags until 48 hr postmortem. Muscles excised at 2 and 3 hr were darker than conventionally processed counterparts.

In order to improve the success of hot boning, researchers have combined ES with HB since ES minimizes the undesirable effects of early postmortem muscle excision and chilling on tenderness (Gilbert and Davey, 1976; Seideman et al., 1979). Taylor et al. (1980) studied the effects of ES and HB on lean color and pigment content. Neither lean color nor total pigment of the semimembranosus muscle were affected by treatment after 5 and 21 days of storage. Hot boning coupled with more rapid chilling produced a more even color across large muscles. This benefit was diminished when ES was incorporated.

Our objectives were to determine the effects of ES and HB, alone and in combination, on display muscle color of polyvinylchloride packaged beef steaks.

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MATERIALS & METHODS

FORTY-EIGHT CROSSBRED STEERS sired by 7/8 Simmental x 1/8 Hereford or Angus bulls and out of crossbred dams were obtained from the R.L. Hruska US Meat Animal Research Center in Clay Center, NE. The cattle were about eight months old and averaged 263 kg when placed on a feeding trial at Kansas State University.

Cattle were fed ad libitum under one of two feeding regimens. First, three accelerated groups (ACC) were stepped-up to a finishing diet over a 58 day period. The final diet (dry matter basis) consisted of 9.6% forage sorghum silage, 84.4% corn, and 6.0% protein and mineral supplement. ACC cattle were slaughtered in three groups after reaching either 441 kg (139 days, ACC 1), 494 kg (178 days, ACC2), or 560 kg (242 days, ACC3). Secondly, the conventionally (CONV) fed cattle were fed a high roughage diet for 110 days, followed by a 21 day pre-finishing adjustment phase and then finished on the same diet as the ACC groups. These cattle were slaughtered after 284 days feeding the same final diet at a mean live weight of 596 kg.

Cattle were slaughtered at the Kansas State University meat laboratory. Carcasses ranged from a group mean yield grade of 2.2 to 3.1, a quality grade of Good 21% to Good 95%, and had average carcass weights of 262 kg (ACC 1), 309 kg (ACC 2), 351 kg (ACC 3), and 358 kg (CONV).

Animals were stunned and bled. Bleeding time was used as time zero for all treatments. Each side was randomly assigned to one of four treatments: control (C), electrical stimulation (ES), hot boning (HB), or electrical stimulation plus hot boning (ESHB).

The C sides were chilled at 5°C until 48 hr postmortem. ES was applied through 6 mm diameter stainless steel probes, one inserted in the inside round about 8 cm below the proximal attachment of the achilles tendon and the other inserted laterally along the humerus. Sides were stimulated 45 min postmortem with 400 to 600 volts of alternating current (60 hertz and 0.6 amp delivered through the carcass) for 2 min with a sequence of 1.6 sec on and 0.8 sec off and chilled until 24 hr postmortem at 5°C. The longissimus (LD) and semimembranosus (SM) muscles from the HB and ESHB sides were excised 2 hr postmortem and stored until 24 hr postmortem at 5°C in an oxygen impermeable bag.

After the cold storage period, one steak (2.5 cm) was cut from both the LD and SM muscles. The LD steaks were cut from over the 2nd to 3rd lumbar vertebral region and the SM steaks were obtained from the distal portion of the SM. Steaks were packaged in conventional 0.8 mil polyvinylchloride film (PVC) and allowed to oxygenate for 2 hr before being displayed continuously (24 hr/day) at 2 – 4°C under General Electric Natural fluorescent (40 watt, 1076 lux) lighting.

Visual appraisal under the display lighting individually done by four member trained panelists and reflectance spectrophotometry were used to evaluate muscle color on days 0 (before light exposure), 1, 3 and 5 of display. Visual scores were estimated to the nearest 0.5 on a five point scale: 1 = bright red, 2 = dull red, 3 = slightly dark red or brown, 4 = dark red or brown, and 5 = very dark red or brown. Reflectance at 474, 525, 580, and 630 nm was measured using a Bausch and Lomb 600 reflectance spectrophotometer adjusted to 100% reflectance with a $MgCO_3$ block.

Reflectance values were used to indicate oxymyoglobin (%R630 nm-%R580 nm), metmyoglobin (%R630 nm/%R525 nm) and reduced myoglobin (%R474 nm/%R525 nm). Additionally, the reflectance values collected at wavelengths 474 nm and 525 nm were converted to K/S values (Francis and Clydesdale, 1975) and the ratio K/S 474 nm / K/S 525 nm was calculated to estimate percent reduced myoglobin (Snyder, 1965).

RESULTS & DISCUSSION

Visual color score

HB steaks (Table 1) from the LD muscles were visually darker ($P < 0.05$) than from all other treatments; except on day 1 of display, HB steaks were not darker ($P > 0.05$) than C steaks (1.7 vs 1.6). HB SM steaks were also visually darker in most comparisons except that HB SM steaks were not different ($P > 0.05$) than ESHB steaks on day 0 and 1, or ES steaks on day 5. Our data agree with those of Kastner et al. (1973) and Kastner and Russell (1975) who found that muscles HB early postmortem (2 - 6 hr) resulted in darker colored muscles than those cold boned at 48 hr. Since the HB muscle may have a tendency to chill faster (Axe et al., 1983), this may alow oxidation and conserve certain biochemical reducing pathways in the muscle. This would help keep muscle in a more reduced state, causing darker appearance. The darker appearance of the HB steaks also may be due, in part, to higher pH (Axe et al., 1983) which results in a higher water-holding capacity (Forrest et al., 1975). Greater binding ability of the proteins and/or a greater structural integrity of the muscle cell membranes as a result of the inhibition of proteolytic enzyme activity may also be involved. With a greater water-holding capacity (i.e. less free water) and a tighter muscle structure, light is absorbed more readily by the muscle tissues, producing a darker appearance (Forrest et al., 1975) and inhibiting oxygen diffusion.

ES LD steaks were brighter ($P < 0.05$) than C LD steaks on day 1 but were not different at the other display times. The ES SM steaks were similar to C steaks in visual color, regardless of the day of display. Mixed results have been reported on the effect of ES on muscle color (Cross et al., 1979; Smith et al., 1979). Grusby et al. (1976) stimulated beef carcasses with 320 volts (5 amps) and found that ES did not affect the color of the LD or SM. McKeith et al. (1980) determined that stimulating with 550 volts was more effective in improving lean color than 150 volt stimulation.

Some of the differences in the effects of ES on color may be accounted for by the differences in the time at which muscles were evaluated. It appears that more color differences are found when color is evaluated soon after death.

The ESHB LD steaks were not different ($P > 0.05$) than C steaks. However, the ESHB SM steaks were darker ($P < 0.05$) than C steaks on days 0 and 1. Evidently, ES alleviated the undesirable effects of HB on the color of the LD but was not as effective in the SM. This might suggest the amount of current reaching the SM, or responsiveness to ES was different from the LD.

Estimated oxymyoglobin (%R630 nm-%R 580 nm)

Following the same pattern established in the visual color score data, the HB LD steaks had lower reflectance difference values (indicating less oxymyoglobin) than all other treatments at all times except for the C steaks on day 0. The SM steaks were somewhat less responsive. However, HB SM steaks were lower in estimated oxymyoglobin ($P < 0.05$) than C and ES steaks except on day 0.

ESHB LD steaks had greater difference values ($P < 0.05$) indicating more oxymyoglobin (brighter) than HB steaks. However, the ESHB SM steaks were not different ($P > 0.05$) than the HB SM steaks. This again suggests that ES was less effective on the SM.

Both ES LD and SM steaks were similar ($P > 0.05$) to counterpart C steaks except on day 0, when ES LD steaks had more oxymyoglobin. Tang and Henrickson (1980) stimulated carcasses for 30 min at 1 hr postmortem, then excised and froze muscles at 4 hr postmortem. LD and

Table 1—Effects of electrical stimulation and hot boning on the visual color score[a] and reflectance measurements of PVC packaged beef steaks

Day	Carcass treatments[b]							
	C	ES	HB	ESHB	C	ES	HB	ESHB
	Visual color score							
	Longissimus				Semimembranosus			
0	1.4^{c}	1.3^{c}	1.6^{d}	1.4^{c}	1.5^{c}	1.3^{c}	1.8^{d}	1.8^{d}
1	1.6^{de}	1.4^{c}	1.7^{e}	1.5^{cd}	1.6^{c}	1.5^{c}	2.0^{d}	1.8^{d}
2	2.0^{c}	1.8^{c}	2.4^{d}	2.0^{c}	2.0^{c}	2.0^{c}	2.6^{d}	2.2^{c}
5	2.5^{c}	2.3^{c}	2.8^{d}	2.4^{c}	2.5^{c}	2.6^{cd}	2.9^{d}	2.6^{c}
	%R630 nm – %R580 nm							
0	24.9^{cd}	27.1^{e}	23.3^{c}	26.2^{de}	25.1^{cd}	25.7^{d}	23.7^{cd}	23.1^{c}
1	23.2^{d}	24.2^{d}	21.1^{c}	24.4^{d}	24.0^{c}	24.0^{d}	21.2^{c}	22.9^{cd}
3	21.5^{d}	22.3^{d}	17.8^{c}	22.0^{d}	22.2^{e}	21.1^{d}	18.2^{c}	19.6^{cd}
5	19.5^{d}	20.0^{d}	15.7^{c}	19.4^{d}	20.0^{d}	19.5^{d}	16.9^{c}	18.7^{cd}
	%R630 nm – %R525 nm							
0	2.72^{cd}	2.89^{e}	2.65^{c}	2.85^{de}	2.91^{d}	2.93^{d}	2.71^{c}	2.73^{c}
1	2.60^{d}	2.57^{d}	2.26^{c}	2.52^{d}	2.83^{e}	2.69^{de}	2.39^{c}	2.61^{d}
3	2.40^{d}	2.34^{d}	2.19^{c}	2.38^{d}	2.58^{d}	2.42^{cd}	2.28^{c}	2.38^{c}
5	2.15^{d}	2.15^{d}	1.93^{c}	2.08^{d}	2.37^{d}	2.31^{cd}	2.20^{c}	2.23^{cd}
	%R474 nm – %R525 nm							
0	1.09^{d}	1.05^{c}	1.08^{cd}	1.06^{cd}	1.05	1.03	1.08	1.05
1	1.02^{c}	1.04^{cd}	1.07^{d}	1.07^{d}	1.00^{c}	1.03^{cd}	1.07^{d}	1.06^{d}
3	1.03	1.05	1.04	1.03	1.03^{c}	1.02^{c}	1.07^{d}	1.04^{cd}
5	1.07	1.05	1.07	1.07	1.02^{c}	1.05^{cd}	1.07^{d}	1.07^{d}

[a] Visual color score: 1 = bright red, 2 = dull red, and 3 = slightly dark red or brown.
[b] C = control, ES = electrical stimulation, HB = hot boning, and ESHB = electrical stimulation plus hot boning.
[cde] Means for the same muscle and row with the same or no superscript letter are not different ($P > 0.05$).

SM muscles from ES carcasses had higher oxymyoglobin content (measured by electrophoresis) than the nonstimulated controls. These carcasses were stimulated for an unusually long period and this may explain why they observed a color difference in the SM. However, Sleper et al. (1983) calculated oxymyoglobin from K/S values and found no difference between L samples from C and ES. They noted higher Hunter CIE "L values" indicating more light scatter for ES samples.

Some of the apparent muscle color differences between treatments may be due to differences in oxygen penetration into the muscle. It has been proposed that ES improves tenderness as a result of some chemical or direct physical alteration in the muscle tissue (Gilbert and Davey, 1976; Savell et al., 1979; Dutson et al., 1980; Judge et al., 1980). Therefore, the color improvement of ES seen early in display suggests that ES may have allowed for more and deeper oxygen penetration, possibly due to a more open structure. However, with additional display time, C steaks may achieve the same level of oxygen penetration, thereby eliminating the color differences.

Estimated metmyoglobin (%R630 nm/%R525 nm)

HB LD steaks had lower reflectance ratio values ($P < 0.05$) indicating more metmyoglobin that all other treatments at all times except C steaks ($P > 0.05$) on day 0. A similar trend ($P > 0.05$) in metmyoglobin accumulation was apparent in the SM; however, there were fewer significant differences.

Metmyoglobin levels were similar between ES and C for the SM steaks at all times and for the LD on days 1, 3 and 5. On day 0, the ES LD steaks had higher reflectance ratio values ($P < 0.05$) than the C steaks which suggests the ES steaks had less metmyoglobin. Also, since reflectance at 630 nm is both a reflectance peak for oxymyoglobin and a reflectance valley for metmyoglobin, a higher ratio of %R630 nm/%R525 nm could indicate more oxymyoglobin, which would support the visual score data.

A greater accumulation of metmyoglobion in HB muscles may be a result of lower oxygen diffusion into the muscle. Brooks (1938) and George and Stratmann (1952) reported that a lower partial pressure of oxygen favors the formation of metmyoglobin. Consequently, a slightly lower amount of oxygen in HB muscle may promote the autoxidation of myoglobin to metmyoglobin.

Estimated reduced myoglobin (%R474 nm/%R525 nm)

Very few differences were found between treatments in the %R474 nm/%R525 nm reflectance ratio for both muscles. Although not statistically analyzed, this reflectance ratio remained fairly constant with display time, while percentage of oxymyoglobin and metmyoglobin changed. Therefore, the reduced myoglobin content was either extremely stable across these treatments and times, or this reflectance ratio is a poor indicator of changes in reduced myoglobin.

Additional reflectance measurements

Conversion of reflectance values at 474 nm and 525 nm to K/S values did not improve sensitivity to changes in reduced myoglobin compared to the ratio of %R474 nm/%R525 nm (data not given). The second estimator of oxymyoglobin (%R580 nm/%R525 nm) did not add any insight to the effects of ES and HB on muscle color as there were fewer differences between treatments.

SUMMARY

HB STEAKS, when compared to C, ES and ESHB steaks, were generally the darkest, had the least oxymyoglobin, and developed more metmyoglobin.

Although ES steaks were statistically similar ($P > 0.05$) to C steaks, they tended to be brighter. Nevertheless, stimulation was sufficient to alleviate the undesirable effects of HB on muscle color.

Regardless of treatment, muscle color was acceptable at all display times of this study and would not present any practical merchandising problems.

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Sensory Considerations in the Replacement in Dark Chocolate of Sucrose by Other Carbohydrate Sweeteners

OLUGBENGA A. OGUNMOYELA and GORDON G. BIRCH

ABSTRACT

Texture, taste, aroma and flavor-related profile descriptors in dark chocolate samples sweetened with different carbohydrates (sucrose, maltose hydrate, B-D-fructose, L-sorbose and sorbitol) were evaluated using a sensory profiling technique. Differences in mouthfeel characteristics of the chocolates were found to reflect the importance of the hedonic component and the relationship between sapid stimulus and physiological response. Most of the sensory differences were found to occur in the profiles of the texture and flavor-related descriptors reflecting the importance of flavor-taste interactions. The results are discussed in terms of the roles of the sweeteners in the acceptance of chocolate sensory qualities.

INTRODUCTION

CHOCOLATE, like most confectionery products, is readily digestible and thus provides easily absorbable energy, which makes a valuable contribution to the nutritive components of the diet. However, with the convincing body of evidence built up by nutritionists in the past two decades, calorie-watching has become almost a compulsion to the extent that many consumers now generally favor nonnutritive sweeteners which virtually pass through the gastro-intestinal tract unaffected (Wingard et al., 1978). Yet, chocolate still retains its attraction primarily because of the desirable flavor and the aesthetic pleasure derived from its consumption.

With the growing controversy over sucrose-related health problems (Yudkin, 1972), and the increasing uncertainty over the safety of artificial sweeteners, many of the so-called 'rare food sugars' are now finding increasing food applications in the chocolate and sugar confectionery industry (Wiggall, 1981) particularly in designed products e.g. noncariogenic and diabetic products. Greater awareness is therefore being generated of the wider spectrum of bulk carbohydrate sweeteners at the disposal of the manufacturer.

Much of the evidence relating high sugar consumption to a number of physiological conditions appears to be mere speculations supported only by epidemiological findings (MacDonald, 1978), but Grenby (1975) has established from investigations with experimental animals, that one of the associated diseases, dental caries, is closely linked to sucrose consumption. As sucrose is the commonest food carbohydrate, he suggested sucrose replacement by other sweeteners as one of the methods of combating dental caries, and noted that this would inevitably involve reformulation of sweet foods and drinks and thus could not easily be applied to all sucrose-containing foods.

The production of greater ranges of chocolate products, utilizing different combinations of sweeteners is now becoming increasingly popular. Owing to the variations in sensory and physicochemical attributes of various sweeteners, these products will be expected to generate variations in quality characteristics as well as manufacturing practices. Indeed, if sucrose consumption should subsequently become restricted, other nutritive sweeteners or combinations filling this role would be expected to provide, in addition to sweetness, the bulk, solubility, crystallinity and a variety of textural attributes for which sucrose has traditionally been employed by the manufacturer.

The objective of this study therefore was to assess the profile sensory differences in dark chocolates prepared using different carbohydrate sweeteners, including sucrose, and thus the relationship between physicochemical properties of different carbohydrate sweeteners and the desirable sensory qualities in chocolate.

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MATERIALS & METHODS

ALL THE SAMPLES of carbohydrate sweeteners used in the study were of analytical grade in order to avoid any unusual taste effects in the prepared chocolates due to the presence of impurities. These were sucrose (obtained from May & Baker Ltd., Dagenham, England), maltose hydrate, B-D-fructose, L-sorbose and sorbitol (obtained from Sigma Chemical Co., England). The 'Bournville' dark chocolate used in this study was obtained from Woolworth Co., Weybridge, England.

Preparation and analysis of dark chocolate samples for sensory testing

The chocolate samples were prepared with the selected carbohydrates under the same conditions in order to ensure that differences in physicochemical attributes of the samples were not due to variations in manufacturing practices. However, slight variations were found necessary in the preparation of chocolates sweetened with B-D-fructose as well as sorbitol, due mainly to their hygroscopic properties. Samples containing maltose hydrate had lecithin and butter oil added at the initial mixing stage in order to obtain a suitable mix and corresponding adjustment was made in the final mixing stage.

Preparation was in small batches, generally as outlined below with slight variations, based on the following ingredient composition: sweetener = 50.0%, cocoa liquor = 45.0%, butter oil = 4.0%, cocoa butter = 0.5% and soya lecithin = 0.5%. The sugar and molten cocoa mass were mixed to a homogeneous mass at 5°C in a Hobart mixer. The mixture was then refined using a small three-roll refiner and the refined flake, transferred to the Hobart mixer, was mixed into a stiff paste at 50°C. Butter oil and cocoa butter were then added and the mixing continued for a further two hours at the same temperature. Lecithin was finally added and mixed in for a further half-hour at 50°C. The resulting chocolate samples were tempered and molded into uniform-sized bars about 10g each. Each of these was cut into equal halves and wrapped in thin foil for storage at 12°C prior to presentation.

Final moisture content, total extractable fat, mean fusion point (°C) and equilibrium relative humidity (ERH) were determined for each of the samples according to Pearson (1976).

Sensory testing/profiling procedure

Ten panelists were selected from a group of 25 on the basis of sensitivity to chocolate flavor differences. A profile technique based on the modified numerical-descriptive scaling procedure recommended by Cartwright and Kelley (1951) was developed for use in this work. Sensory profile descriptors and numerical scales outlined on questionnaires presented to panelists for the evaluation of the test samples were constructed from the glossary compiled by the ten panelists at initial trials in which 'Bournville' dark chocolate of similar appearance as the test samples, but of unknown composi-

tion, was examined. Selection of these descriptors was based primarily on clarity of expression, relevance to the desired objectives, and lack of repetitiveness and subjectiveness of measurement.

In evaluating the test samples, no special lighting was required. The samples were coded in such a way as to be unidentifiable to each of the panelists. Panelists were requested to pause for 60 sec between samples.

Boiled and cooled tap water was presented to panelists for rinsing between samples during taste sessions. By controlling the number and size of samples at each sitting, flavor carry-over effects and panelist fatigue, which may reduce individual sensitivity to flavor differences, were eliminated.

Details of procedures to be adopted by the panelists for the assessment of each descriptor were discussed and agreed upon at the initial trials in order to ensure uniformity both in the meaning of the terms used and the methods by which they were evaluated, thus minimizing within and between panelist variations.

Three final profile sessions were held on each sample and the results of these three sessions were averaged arithmetically to establish the final profile data subjected to statistical treatment.

Sweetness was assessed using the numerical scale 0-10 where 0 = no sweetness and 10 = extremely sweet. Flavor and aroma characters were assessed on a six-point numerical-descriptive scale where 0 = not detectable, 1 = just detectable, 2 = moderate, 3 = moderately strong, 4 = strong and 5 = very strong. Textural parameters were evaluated by category scales of varying magnitudes depending on the attribute concerned, each based on the general concensus of panelists, (e.g. nine-point numerical-descriptive scale for hardness where 1 = extremely soft and 9 = extremely hard, similarly five point scale for 'adhesiveness' and seven-point scale 'chewiness').

'Melt time' (sec) was estimated as a mouthfeel characteristic related to the moisture and fat contents of the samples, and taken as the time for a standard 5-g sample to melt completely into a flowing mass when held in the mouth, as determined with a stopwatch.

It was observed that a pleasurable sensory experience resulted from chocolate tasting and that the enhanced physiological response of increased salivary secretion varied from sample to sample. Thus, the degree of 'ensalivation' induced by the test samples when chewed in the mouth was evaluated on a nine-point numerical-descriptive scale with 'little or no ensalivation' and 'extreme ensalivation' anchoring both ends of the scale.

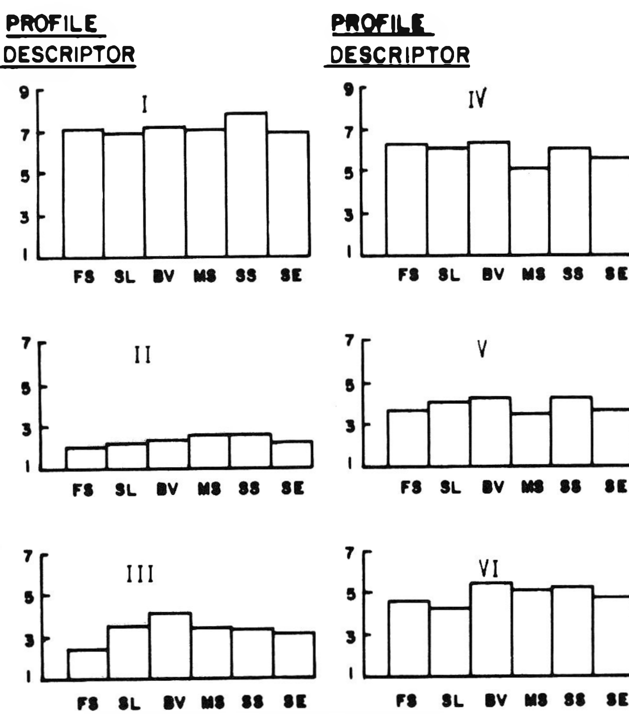

Fig. 1—Texture profile descriptors I-VI are as follows:

(I) SENSORY HARDNESS: evaluated on a 1-9 category scale. Standard error of difference between two means (SEM = 1.24). (II) ADHESIVENESS: evaluated on a 1-5 category scale (SEM = 0.87). (III) CHEWINESS: evaluated on a 1-7 category scale (SEM = 1.14). (IV) ENSALIVATION: evaluated on a 1-9 scale (SEM = 2.11). (V) ORAL VISCOSITY: evaluated on a 1-7 scale (SEM = 2.14). (VI) ORAL MELT TIME (x10), evaluated in seconds (SEM = 1.44). Means of 15 judgments are presented. FS = B-D-fructose, SL = sorbitol, BV = Bournville, MS = maltose hydrate, SS = sucrose, and SE = L-sorbose.

Experimental design

Because of the limitations imposed by quantities of prepared samples available, a Balanced Incomplete Block design (Brownlee, 1957) was used, thus giving six treatments (samples) in ten blocks (panelists) of three units (i.e. three out of six samples were evaluated by each panelist), resulting in five replications per sample.

Each panelist, therefore, evaluated three samples at three different sittings in a balanced, pre-determined order of presentation (e.g. abc, bca and cab respectively) thus ensuring that each sample was tested exactly the same number of times but no panelist evaluated all the samples. The limitation of this design is normally the requirement of fairly large numbers of panelists for increasing accuracy but its greatest advantage is in ensuring that panelist fatigue and flavor carry-over effects are eliminated as a result of limited sample size.

The arithmetic averages were analyzed statistically according to the analysis of variance technique of Yates (Brownlee, 1957). The relative weightings of the flavor descriptors were also subjected to multivariate analysis of variance (Morrison, 1967) to assess significance of variations between panelists and sweeteners.

RESULTS & DISCUSSION

ON EACH CHOCOLATE SAMPLE, the means of 15 judgments (five panelists x three different orders) and the standard error of difference between the means (SEM) were calculated for each descriptor. Means of the six samples obtained for each descriptor did not appear to show wide fluctuations in many cases, and sensory profiles were thus drawn from these values for a ready comparison of the important differences between the samples.

The individual profile descriptors for each of the chocolate samples were presented as histograms as shown (Fig. 1 for texture-related descriptors; Fig. 2 for aroma intensity

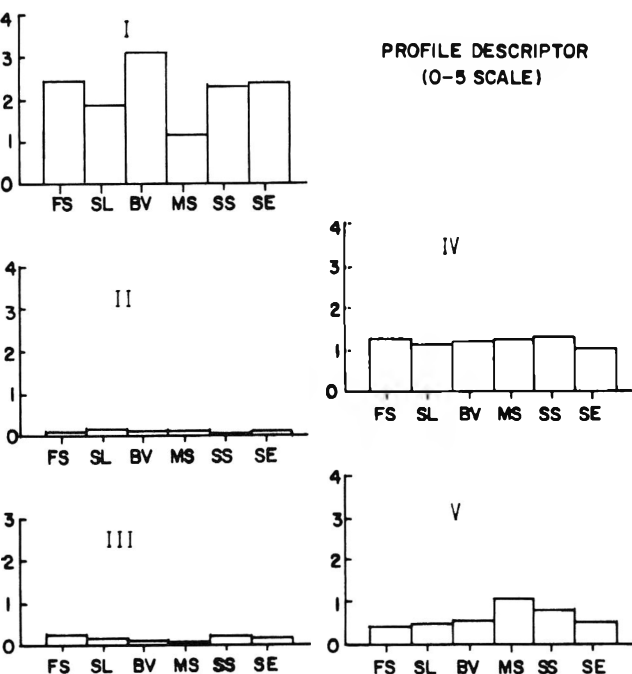

Fig. 2—Aroma profile descriptors I-V are as follows:

(I) OVERALL FLAVOR/AROMA: evaluated on a 0.5 category scale as all other profile characters. Standard error of difference between two means SEM = 0.99. (II) = COCOA FAT: SEM = 0.37. (III) = SWEET: SEM = 0.76. (IV) = CHOCOLATE: SEM = 1.07. (V) = BITTER: SEM = 1.40. Means of 15 judgments are presented. The symbols FS, SL, BV, MS, SS and SE are as outlined in legend to Fig. 1.

notes and Fig. 3 for flavor intensity notes), thus allowing adequate comparisons to be made with respect to the profiles of the different samples.

Table 1 also shows the differences between the samples due primarily to the sweeteners. While not much difference existed between the samples in the total extractable fat, the samples differ in final moisture content, particularly B-D-fructose and maltose hydrate. Sorbitol-sweetened chocolate had a higher fusion point than the other samples, indicating its suitability for the manufacture of tropically designed chocolates. B-D-fructose-sweetened chocolate was found to have a higher ERH than the others which probably reflects the hygroscopic nature of the sweetener. It is therefore inevitable that chocolate manufacture with B-D-fructose requires a strict control of temperature and humidity in order to reduce moisture uptake.

Texture-related parameters

The use of the profile technique for assessing the relative importance of texture-related descriptors in the alteration of perceived stimulus has not hitherto been investigated. Yet it is known that the physical state of a food may affect its taste since texture may partially control the rate at which sapid stimuli accede to the taste cells, and when added to foods and beverages, the relative sweetness of different sweeteners varies according to differences in their textures (Crocker, 1945). From Fig. 1, chewiness of B-D-fructose was significantly different ($p < 0.001$) from that of other chocolate samples. This is probably attributable to the high hygroscopicity of B-D-fructose.

On the basis of the degree of ensalivation induced by the samples, panelists judged the maltose hydrate-sweetened sample to cause less ensalivation, although not significantly less, than other samples. This is an attribute that is seldom measured but reflects the differences in mouthfeel due to the different sweeteners. It is an important hedonic component which indicates the close relationship between sapid stimulus and physiological response, the maltose hydrate-sweetened chocolate being the least sweet of all the samples considered here. The oral melt time was found to correlate well with oral viscosity ($r = 0.72$), molecular weight of the sweeteners ($r = 0.64$), sweetness ($r = 0.66$) and sensory hardness and degree of ensalivation ($r = 0.58$). This indicates that it interacted with many chocolate attributes that constitute the hedonic component.

Sweetness

Table 2 lists the mean sweetness values for the sweetened chocolate samples. Sweetness of the B-D-fructose, L-sorbose, sorbitol- and sucrose-sweetened samples (b) were of similar magnitude and significantly different from the maltose hydrate-sweetened chocolate (a) ($p < 0.001$). The low sweetness of the maltose hydrate-sweetened sample reflected the low relative sweetness of that carbohydrate. The inability of panelists to detect sweetness differences between B-D-fructose, sucrose, L-sorbose and sorbitol may be due to the high sweetness masking in dark chocolates. It is thus possible that in a milder flavored system like milk chocolate, the sweetener differences may become very apparent.

Although B-D-fructose is the sweetest sugar known, the sweetness intensity of its chocolate sample was found to be lower than those of sorbitol, L-sorbose and sucrose. Although the difference was not statistically significant, the depression of sweetness intensity of B-D-fructose reflected the basis difference between the sweetness of the sapid compounds in water and in food products. This is consistent with the observation of Mackey and Valassi (1956) that threshold values of sapid compounds in water were lower than in foods and that texture appeared to affect the ease of discernment of added sweet, sour or salty substances. If synergism or suppression effects are of significance in foods, as in aqueous sugar mixtures (Bartoshuk, 1975), due to the interaction of food components, then the sweetness effects observed, e.g. apparent enhancement of sorbitol sweetness and suppression of fructose sweetness, relative to sucrose, are probably attributable to these effects. Thus estimations of sweetness effects of different carbohydrate sweeteners, based on the relative sweetness of the sweeteners alone or of those sweetener mixtures in aqueous systems, would undoubtedly be inappropriate for prediction of sweetness in chocolate and confectionery.

Sweetness intensity ratings were found to correlate very highly with the degree of ensalivation ($r = 0.96$). This confirms that salivary secretion, which is physiologically controlled, is an important indicator of a pleasurable experience during gustation.

Flavor-related parameters

According to Solms (1969), sugars round and blend flavor components and provide mouthfeel effects. Although, these are secondary effects (Wasson, 1969) sugars often cause pronounced flavor effects in some confectionery products. However, Fig. 2 shows that there were no significant differences in the odor components or aroma profile descriptors of the different chocolate samples which were ascribable to the sugars. Maltose hydrate-sweetened chocolate had a higher rating than other samples in the 'bitter' note of its odor component. This probably reflects the outstanding sensitivity of the bitter quality due to flavor-taste interaction, since maltose hydrate was the least sweet of the sweeteners under consideration. Significant differences were found between the samples in terms of the overall amplitude rating which also exemplify the importance of flavor-taste interaction. Maltose hydrate sweetened chocolate was significantly different from other samples ($p < 0.001$) while sorbitol was less significantly different ($p < 0.005$).

Fig. 3 shows the differences among the flavor profile descriptors of the chocolate samples. Differences among the samples due to the different carbohydrate sweeteners were mostly evident in the taste component of the flavor. The

Table 1—Analytical data on some important physical-chemical parameters in sample dark chocolates

Sweetener	Total extractable fat (%)	Final moisture content (%)	Mean fusion point (°C)	ERH (%)
Sucrose	31.08	0.85	32.5	32
Maltose hydrate	30.57	3.83	30.5	33
B-D-fructose	30.04	6.03	30.3	45
L-sorbose	30.32	1.21	32.6	34
Sorbitol	28.73	1.05	39.5	35
Bournville	28.08	0.54	31.7	33

Table 2—Mean sweetness intensities of sweetened dark chocolate samples. Means of 15 judgments are presented (Standard Error of difference between two means = 2.00)

Sweetener	Mean sweetness intensity (0–10 scale)	
Maltose hydrate	1.62	(a)
B-D-fructose	4.69	(b)
L-Sorbose	4.80	(b)
Sorbitol	4.82	(b)
Sucrose	4.96	(b)

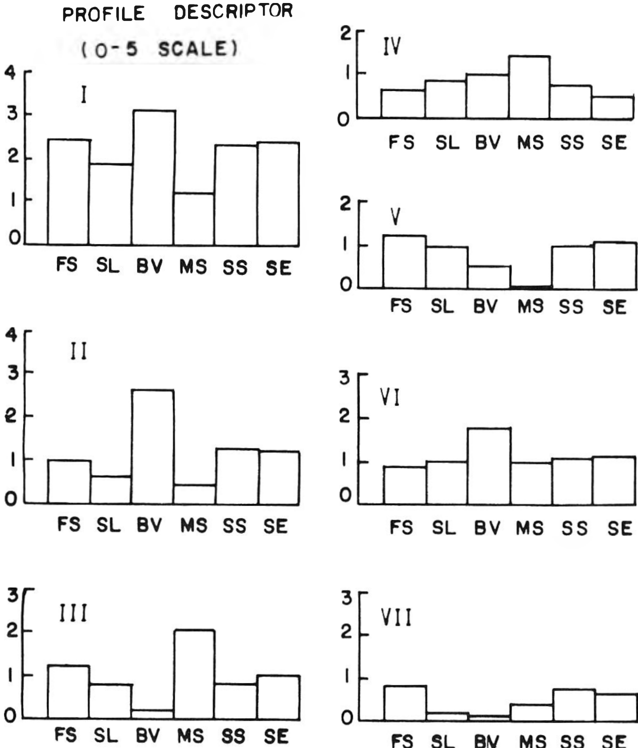

Fig. 3—Flavor profile descriptors I–VII were all evaluated on a (6-point) category scale and are described below:

(I) OVERALL FLAVOR/AROMA: standard error of difference between two means (SEM) = 0.99. (II) SWEET: SEM = 1.12. (III) BITTER: SEM = 1.75. (IV) COCOA FAT: SEM = 1.69. (V) BITTER-SWEET: SEM = 1.20. (VI) CHOCOLATE: SEM = 0.98. (VII) COCOA ROAST: SEM = 0.94. Means of 15 judgment are presented. The symbols FS, SL, BV, MS, SS and SE are as outlined in legend to Fig. 1.

sweet and bitter components of the flavor were significantly different ($p < 0.001$) for the maltose-hydrate sweetened chocolate, compared with other samples, reflecting the same trend of sweetness scores observed in Table 3. This explains why it is generally believed that the combined sensations of taste and odor lead to flavor (Moncrieff, 1967), although the perceived flavor may be modified by mouth effects causing changes in the order of appearance of the character notes as well as a modification of the after-taste of flavor (Wasson, 1969). This significance of the sweeteners in the observed flavor is well illustrated in Fig. 3. Notably prominent ($p < 0.001$) differences were found in the taste-related descriptors, namely 'sweet', 'bitter-sweet' and 'bitter', illustrating the differences in relative sweetness of the sugars.

When the six flavor descriptors evaluated were subjected to multivariate analysis using the method of Morrison (1967) to assess the significance of differences between panelists and sweeteners and the relative contribution of each flavor note or combination of flavor notes to the recognition of such differences, it was found (Table 3) that both sets of variations were significant ($p < 0.001$). Considering the relative weightings of the six descriptors accounting for unidimensional variations between both panelists and sweeteners (Table 4), most of the variations were found between panelists. It was noted that variations in terms of the 'sweet' component were higher between the sweeteners than between panelists, probably due to the synergistic effects of flavor-taste interactions. It appears that considerable differences exist in the ways individuals react to the chocolate flavor note, perhaps reflecting the variations in mouthfeel characteristics of the samples for

Table 3—F-statistics for significant differences between panelists and sweeteners

Source of variation	df	F-Statistic
Between panelists	54,56	3.74***
Between sweeteners (eliminating panelists)	30,42	2.59***

Table 4—Relative weightings of profile parameters for unidimensional variations between panelists and sweeteners

Flavor profile parameter	Relative weightings: Differences between panelists	Differences between sweeteners
Chocolate	2.281	1.631
Sweet	0.315	0.854
Fat	0.892	0.622
Bitter-sweet	−0.870	−0.896
Bitter	0.881	0.149
Roast	1.228	0.617

individual panelists. Thus 'chocolate' accounted for most of the observed variations both between panelists and between sweeteners, apparently confirming the complicating effects of sugars on this flavor note.

The 'sweet' component of the flavor gave a high correlation with the overall amplitude rating ($r = 0.96$) and reflects the importance of the taste component in the overall acceptability of the flavor of chocolate. Both the 'bitter' and 'bitter-sweet' components also gave some correlation with the 'fat' component ($r = 0.88$ and $R = 0.69$ respectively) thus confirming that fats exert considerable indirect taste effects due to their lipophilic properties (Forss, 1969) which is evident here primarily because of the high sensitivity and lingering nature of bitterness.

CONCLUSION

THE USEFULNESS of the sensory profiling technique in the appraisal of sensory qualities of chocolate products cannot be over-emphasized from the foregoing considerations. Because of the variations in their physicochemical properties, different carbohydrate sweeteners show wide variations in their sensory effects in chocolate, especially with respect to taste and flavor and thus the hedonic quality of the products.

Sorbitol is already used for special chocolate designs, e.g. diabetic products, but it is unlikely that such products can replace sucrose-sweetened chocolates. However, L-sorbose, which compares favorably with sucrose, may be a promising carbohydrate to provide the many advantages desired in chocolate products such as texture, flavor and sweetness, as well as reduction of dental caries, since it is not fermented by oral bacteria.

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A New Method for Sensory Evaluation of Red Pepper Heat

M. H. GILLETTE, C. E. APPEL, and M. C. LEGO

ABSTRACT

A replacement for the Scoville heat test for the sensory determination of pungency in capsicum products has been developed. Ground red pepper was steeped in 90°C water for 20 min, filtered and the filtrate diluted in 20°C water. Trained panelists compared the heat of pepper extracts with a known concentration of a standard, synthetic capsaicin, (N-vanillyl-n-nonamide). The reproducible results correlated highly with high pressure liquid chromatography.

INTRODUCTION

THE TERM "CAPSICUM" refers to the fruit of numerous species of the genus capsicum. These fruits vary widely in size, shape, flavor and sensory heat, Included in this genus are: red pepper (also called cayenne or chillies), paprika, and sweet peppers. The food industry is the largest user of capsicums, where the spice is used as a coloring and flavoring agent in sauces, soups, processed meats, snacks, candies, soft drinks and alcoholic beverages (Mathew et al., 1971) either in the ground form, or as an oleoresin (concentrated extract).

The chemical compound "capsaicin" is the primary contributor of heat to capsicums. Other compounds, structurally similar to capsaicin, also contribute to the heat of red pepper. This family of compounds, including capsaicin, is termed "capsaicinoids."

In order to produce a consistent product, the heat level of capsicums is monitored by sensory or chemical/instrumental methods. The most commonly used sensory method for determining heat in capsicum products is the Scoville Heat Test (Scoville, 1912) which has been adopted, with modifications, by the American Spice Trade Association (ASTA) Analytical Method 21.0 and by International Organization for Standardization (ISO). Other modifications to the Scoville Method have been proposed by Govindajaran et al. (1977), Todd et al. (1977) and Rhyu (1978).

The Scoville Method has been severly criticized (Suzuki et al., 1957; Maga, 1975; Govindajaran et al., 1977; Todd et al., 1977; Rhyu, 1978) but continues to be employed as the only sensory assessment of capsicum heat. Specific problems noted with the Scoville Heat Test are: build up of heat, rapid taste fatigue, increased taste threshold, ethanol bite in the samples, lack of statistical validity, lack of reference standards, extraction time (16 hr), poor reproducibility, and the error of central tendency. Chemical and instrumental methods for monitoring the chemical heat in capsicum are being developed with increased frequency (Masada et al., 1971; Di Cecco, 1976; Palacio, 1977; Sticher et al., 1978; Bajaj, 1980). These values are often converted to Scoville Heat Units for expression in a sensory, rather than chemical, mode.

The purpose of this study was to develop a sensory method for the evaluation of heat in ground red pepper as a replacement for the Scoville method of pungency determination, for use in the quality control of red pepper heat, and for the accurate evaluation of chemical/instrumental measurements of red pepper heat.

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MATERIALS & METHODS

Sample preparation

To test the accuracy of the new method, a series of 15 "artificial" red peppers of known heat principle concentration were prepared by blending varying levels (0-12.5%) of oleoresin capsicum on ground paprika. The 15 samples were tested by high pressure liquid chromatography (Hoffman et al., 1983) for homogeneity. A 35-g aliquot of each "pepper" sample was removed for further chemical and sensory evaluation.

Five grams of each artificial pepper sample were added to 1995g "Polar" brand spring water at 20°C, extracted for 20 min at 20°C, filtered through "Mr. Coffee" brand filter paper, and diluted tenfold in spring water at 20°C (20g filtrate in 180g water).

A standard, or "control" sample was prepared using a ground red pepper known to have a "slight" amount of heat (20,000 Scoville Heat Units); 0.25g of this pepper was added to 99.75g spring water at 20°C and extracted, filtered, and diluted as above.

To evaluate the extraction procedure, ground red pepper was extracted with one of the following: (1) spring water at 20°C; (2) spring water at 20°C plus 20-2000 mg/L Polysorbate –80; (3) spring water at 75°C; (4) spring water at 75°C plus 20-200 mg/L Polysorbate –80; (5) spring water at 75°C plus Polysorbate –80 and a 20 min 90°C simmer; (6) 95% ethanol.

When the polysorbate –80 was used, it was blended directly with the red pepper. All aqueous dilutions were prepared as described above for the "control" sample. For the ethanol extraction, 0.50g of pepper was placed in a flask with 100 mL of 95% ethanol, a condensor attached, and the material was stirred at 55-60°C for 5 hr. A 50 mL aliquot of the supernatant was transferred to a 100 mL flask and concentrated under vacuum to yield 0.086g of residue. The residue was diluted to 100 mL with water at 20°C and, after 20 min, filtered through coffee filter papers. The filtrate was diluted 10-fold with spring water at 20°C.

The final calculated concentration of pepper was 250 mg/L, the same as for the aqueous extractions. The residue remaining on the filter papers was re-extracted with ethanol and water as described above and evaluated for remaining sensory heat.

To correlate sensory and instrumental responses, samples from 60 lots of ground red pepper were selected to represent the normal range of Scoville Heat Units found in red pepper. Scoville Heat Values for these 60 peppers were determined by ASTA method 21.0. Using the new method, samples (0.25g) of each lot and 0.02g Polysorbate –80 were diluted to 100g with spring water at 75°C and simmered at 90°C for 20 min on a Corning Hot Plate Stirrer PC-351, with stirring. The extracted pepper was filtered through coffee filter papers and 10g of the filtrate diluted to 200g with spring water at 20°C. Final concentration of extracted pepper was 125 mg/L and 10 mg/L Polysorbate –80.

To replace the ground red pepper "control", N-vanillyl-n-nonamide (N-[(4-hydroxy-3-methoxyphenyl)methyl]-n-nonamide) was selected as a standard reference for the sensory evaluation of red pepper heat. A series of solutions containing varying amounts of N-vanillyl-n-nonamide was prepared by adding 0.6g of the chemical to 20g Polysorbate –80, blending well and diluting to 1 liter with spring water at 20°C. Ten ml of this solution were further diluted to 1 liter with spring water at 20°C, and then to concentrations ranging from 0.11 mg/L to 1.32 mg/L N-vanillyl-n-nonamide.

Sensory evaluation

For development of the method, a 12 member trained panel, experienced in the method of measurement and in the evaluation of

pepper heat, was used in all sensory tests. Pepper heat was rated on a 15 cm line scale anchored at 0 (no heat), 1.25 cm (threshold heat), 5.0 cm (slight heat), 10.0 cm (moderate heat), and 15 cm (strong heat). The tests were conducted in a descriptive panel room on a round table. Red lights were used to eliminate the possible effect of variation in the color of the products. Two samples were evaluated per test, the known control and a pepper extract. The control, coded "C" was served first followed by an unknown test sample identified with a random double letter code. All samples were presented as 10 ml portions in plastic medicine cups. Panelists evaluated all samples using the following procedure:

1. Cleanse palate before first sample (control) with unsalted cracker and 20°C spring water.
2. Take entire 1st sample (control) in mouth, hold for about 5 sec, swallow slowly.
3. Wait 30 seconds (timed).
4. Rate 1st sample at "slight" on ballot.
5. Cleanse palate with unsalted cracker and 20°C spring water for 60 seconds (timed).
6. Rinse with 20°C spring water immediately prior to 2nd sample.
7. Take entire 2nd sample (test sample) in mouth, hold for about 5 seconds, swallow slowly.
8. Wait 30 seconds (timed).
9. Rate 2nd sample.
10. Panel dismissed if only one sample is to be evaluated. If two samples are to be evaluated:
11. Wait 5.0 minutes. Cleanse palate well with water and crackers during this time.
12. Repeat steps 1 through 9 for the second set of samples.

Panelists placed a mark on the scale expressing their impression of the heat in the test sample. Sensory Heat Ratings were obtained by measuring the distance in cm from the "0" mark to the panelist's rating for each sample. The mean of all panelists ratings for each sample represents its sensory heat rating.

For evaluation of the 60 natural peppers, two sets of samples were evaluated per session.

An interlaboratory study was conducted using five panels in three separate intercompany laboratories. The laboratories each evaluated six ground red pepper samples following the new method as described above.

Chemical analyses

The capsaicinoids capsaicin (N-[(4-hydroxy-3-methoxyphenyl) methyl]-8-methyl-6-nonenamide), nordihydrocapsaicin (N-[(4-hydroxy-3-methoxyphenyl)methyl]-7-methyl-octanamide) and dihydrocapsaicin (N-[(4-hydroxy-3-methoxyphenyl)methyl]-8-methyl-nonamide) were extracted from the ground red pepper with 95% ethanol, then separated by reverse phase HPLC, identified by their absorption at 280 nm and quantitated relative to an external standard (N-vanillyl-n-nonamide) according to the method developed by Hoffman et al. (1983). These quantitative measures were combined with the individual capsaicinoid sensory values determined by Todd et al. (1977) to obtain "Calculated Scoville Heat Units" (Hoffman et al., 1983).

Moisture content of the samples was determined on 25g of ground red pepper by azeotropic distillation (American Spice Trade Association Method #2.0), and by vacuum oven (American Spice Trade Association Method #2.1). The values from the two methods were averaged and reported as a mean value.

The surface color (L, a, b, ΔE) of the ground red pepper was measured directly on a Hunter Colorimeter model D25M-9. The samples were placed in a sample cup having an optically clear glass base and the sides protected from light by black tape. This was placed on top of the optical sensing device and the measurements made and recorded automatically.

Water activity of the samples was determined in duplicate at 25 ± 0.1°C using a Beckman Sina Hygrometer-Hygroline (Model #8-0011). Standard salt calibration samples were run simultaneously.

Particle size distribution (μ) and surface area (m^2/cm^3) were measured using an ATM sonic sifter (Model #L3-P) and screens of 420μ, 297μ, 210μ, 149μ, 105μ, and 74μ.

Statistical analysis of data

The sensory data were analyzed by ANOVA and Duncan's Multiple Range Test. For the "artificial" red peppers, the sensory data and the percent oloeresin capsicum were analyzed by linear and curvilinear regression analysis.

For the first 40 of the 60 ground red peppers evaluated, all possible single and multiple regression analyses were performed using the sensory data as the dependent variable and the analytical data including "calculated" Scoville Heat Values (Hoffman et al., 1983) as independent variables. The data on the remaining 20 red pepper samples were used to test the predictive strength of the regression equations. The regressions were also calculated on the entire set of 60 ground red peppers. Additionally, response surface methodology (CompuServe, CS-307, 1978) was performed on sensory vs % capsaicinoids and moisture content and on sensory vs % capsaicinoids and water activity of the 60 peppers.

For the interlaboratory study, laboratory means and standard deviations were calculated, as well as inter-lab means, standard deviations and coefficients of variation, for the six ground red peppers tested.

RESULTS & DISCUSSION

THE SENSORY HEAT VALUES of the artificial red peppers were highly correlated ($r^2 = 0.92$) with the percent oleocapsicum in the "artificial" set of red peppers (Fig. 1).

The r^2 value indicates that 92% of the variation in the sensory heat values can be explained by the variation in oleocapsicum content. The equation relating Sensory Heat Value to Percent Oleocapsicum is: Sensory = 1.16 (% Oleoresin) + 1.10. The relationship between sensory heat value and percent oleoresin was linear within the range tested.

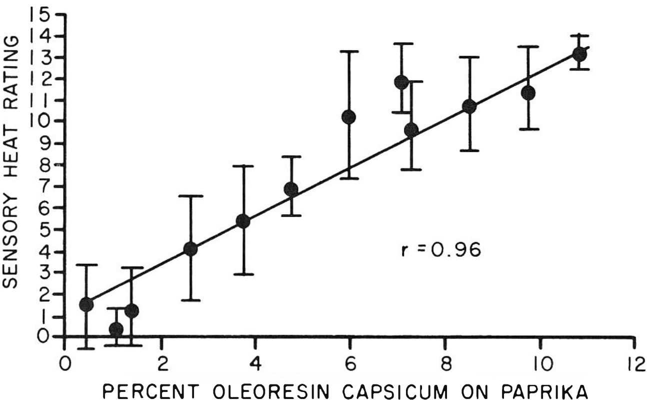

Fig. 1—Sensory heat ratings versus concentration of oleoresin capsicum on paprika.

Table 1—Means and standard deviations for sensory heat ratings of 15 artificial[a] red peppers

% Oleocapsicum	Sensory heat rating	95%*	99%**
12.5	14.2 ± 0.8	a	a
11.5	13.0 ± 1.5	ab	abc
10.4	13.4 ± 0.7	ab	ab
9.4	11.6 ± 2.0	cde	bcde
8.2	10.9 ± 2.2	def	cde
7.1	9.9 ± 2.3	fg	de
6.9	12.0 ± 1.7	bcd	abc
5.8	10.4 ± 1.7	defg	de
4.6	7.0 ± 1.4	h	e
3.6	5.5 ± 2.6	i	ef
2.6	4.2 ± 2.5	i	f
1.2	1.3 ± 1.7	j	g
1.0	0.5 ± 0.9	j	g
0.4	1.5 ± 1.8	j	g
0	0.8 ± 0.9	j	g

[a] Artificial Red Pepper = Paprika plated with varying levels of oleoresin capsicum.
* Means followed by the same letter are not significantly different at the 95% level of confidence.
** Means followed by the same letter are not signifcantly different at the 99% level of confidence.

There was no significant difference among panelists; therefore, the panelists were reacting in a similar manner to each sample. Perhaps this particular group was behaving in a cohesive manner due to the training, experience, and/or the presence of a reference sample.

There were significant differences in the sensory heat ratings among the 15 samples of artificial red pepper (Table 1). The 15 samples can be clustered into four groupings: a threshold heat group (1.3 < % oleocapsicum); a weak heat group (2 < % oleocapsicum < 6); a moderate heat group (7 < % oleocapsicum < 10) and a strong heat group (10 < % oleocapsicum < 12). Scoville Heat Units (S.H.U.) calculated from the capsaicinoid content of the samples (Hoffman et al., 1983) were: approximately 25,000 for the "slight" rating; 50,000 for "moderate;" and 75,000 for "strong."

Table 2 summarizes the results of the various solvent and extraction procedures for the heat principles in red pepper. The use of 20 ppm polysorbate-80 significantly ($p \leqslant 0.05$) increased the amount of heat extracted from red pepper in water at 20°C. A simmering extraction, with or without polysorbate-80, provided significantly more sensory heat than a 20°C extraction. The melting point of capsaicin has been reported to be about 64°C (Newman, 1953), which may explain why more heat was extracted with hot rather than with room temperature water. Ethanol did not extract any more sensory heat from red pepper than the hot water/polysorbate-80 extraction did. After extracting red pepper with simmering 90°C water and 20 mg/L polysorbate-80, the filtered residue yielded only threshold sensory heat on re-extraction with water or ethanol.

Sensory heat ratings for varying concentrations of N-vanillyl-n-nonamide are shown in Table 3. A concentration of 0.44 mg/L was selected to represent "slight" (5 cm), 0.77 mg/L to represent "moderate" (10 cm), and 1.32 mg/L to represent "approaching strong" (13 cm) pepper heat on the 15 cm line scale. Since taste thresholds vary significantly between people, the threshold level was defined by concept rather than by attempting to find one dilution to represent a threshold concentration of pepper heat for all panelists. Similarly, it was considered desirable to leave room at the "strong" end of the scale for an unusually hot red pepper sample and define "strong" by concept only.

The analytical and sensory data collected on the 60 ground red peppers are shown in Table 4. Excellent correlations were obtained between the sensory data and the analytical data gathered on the natural ground red peppers (Table 5).

For predictive purposes, the independent variable "% capsaicinoids" (Fig. 2) was selected as the most desirable indicator of sensory heat, as only one measurement need be taken to predict sensory heat. The predictive strength of the regression equation relating sensory heat to percent capsaicinoids was good; the mean average deviation between actual and predicted sensory values was 0.78 cm on the 15 cm line scale (Table 6). Thus, the regression equation "Sensory Heat = 31.26 (% capsaicinoids) − 0.21" accurately predicts sensory heat via total capsaicinoid content of the pepper.

No correlation was found between the laboratory determined Scoville heat units and the sensory heat ratings ($r = 0.40$,) or between the laboratory determined Scoville heat units and the % capsaicinoids ($r = 0.48$,) of the 60 red peppers. An excellent correlation was found between the sensory data and the calculated S.H.U. Using this correlation, the sensory heat rating scale was divided into seven classifications of heat levels (Fig. 3). This enables description of the pepper with a verbal rather than numerical description of the heat level.

The RSM analysis indicated that 89.8% and 89.9%, respectively, of the variation in sensory values could be ex-

—Text continued on page 1032

Table 2—Sensory heat ratings for ground red pepper extracted by several means

Test #	Extraction method[a]	Sensory heat rating
1	20°C Spring Water[b]	5.0
	20°C Spring Water + 20 mg/l Polysorbate-80	6.6*
2	20°C Spring Water	7.1
	75°C Spring Water	9.1*
3	75°C Spring Water	8.3
	90°C Simmering Spring Water	13.4**
4	20°C Spring Water + 20 mg/l Polysorbate-80	7.5
	90°C Simmering Spring Water + 20 mg/l Polysorbate-80	13.3**
5	20°C Spring Water	7.5
	Ethanol	7.0
6	20°C Spring Water	5.2
	Ethanol	5.0
7	Ethanol extraction of residue from ethanol extraction	1.25[c]
8	Ethanol extraction of residue from ethanol extraction	1.0
9	Ethanol extraction of residue from aqueous extraction	2.4[d]
10	Ethanol extraction of residue from aqueous extraction	2.2[d]
11	Aqueous extraction of residue from aqueous extraction	2.2[d]

[a] Ground red pepper was extracted using the solvents and procedures described. For the aqueous extractions, 0.25 g of pepper was diluted to 100 g with water, stirred for 20 minutes, filtered and diluted 20-fold with 20°C water. For the ethanol extraction, 0.50 g of pepper was extracted with 100 ml of 95% ethanol at 55° - 60°C for 5 hours. 50 ml of this was concentrated to 0.086 g residue, diluted to 100 ml with water at 20°C, and after 20 min. filtered and diluted 20 fold with 20°C water.
[b] Polar Brand Spring Water
[c] Significantly different from plain water at the 95% level of confidence.
[d] Significantly different from plain water at the 99% level of confidence.
* Significantly different from other sample in test at the 95% level of confidence.
** Significantly different from other sample in test at the 99% level of confidence.

Table 3—Sensory heat ratinge for varying levels of n-vanillyl-n-nonamide

Mg/L N-Vanillyl-n-nonamide	SHR[a]
0.11	1.0
0.22	1.0
0.22	1.1
0.40	3.6
0.44	4.8
0.50	6.4
0.60	8.1
0.66	8.3
0.77	7.9
0.77	8.9
0.77	10.6
0.88	11.3
0.88	11.0
1.32	13.2

[a] Sensory Heat Rating; 1.25 = threshold, 5.0 = slight, 10.0 = moderate, 15.0 = strong. Average rating from 10 member trained panel.

Table 4—Analytical and sensory data collected on 60 samples of ground red pepper

Pepper sample code	Surface area[a]	Mean part size[b]	Mois[c]	a_w[d]	% Nor[e]	% Cap[f]	% Dihy[g]	% Capn[h]	Calc Scov[i]	L[j]	a[j]	b[j]	ΔE[j]	Scov[k]	Sensory heat[l]
C-00-154	0.020	344	5.35	3.70	0.005	0.047	0.017	0.069	10,769	36.5	28.0	21.1	50.63	28,000	1.02
C-00-155	0.021	336	5.31	3.84	0.014	0.177	0.082	0.273	43,001	40.6	26.0	23.7	53.69	40,000	8.48
C-00-175	0.018	356	6.17	4.99	0.027	0.257	0.100	0.384	59,988	39.2	31.2	23.3	55.23	62,000	11.25
C-00-202	0.021	314	6.54	4.99	0.019	0.207	0.079	0.305	47,813	40.4	18.7	22.3	49.79	46,666	10.06
C-00-147	0.018	376	4.03	3.28	0.015	0.181	0.088	0.284	44,704	35.4	23.9	19.8	47.00	75,000	10.13
C-00-172	0.019	360	4.21	3.70	0.006	0.049	0.026	0.081	12,633	38.1	29.3	22.4	53.00	31,000	1.37
C-00-179	0.019	344	6.08	4.98	0.030	0.277	0.111	0.418	65,258	40.5	29.5	24.0	55.54	63,500	12.60
C-00-156	0.020	346	4.76	3.52	0.009	0.082	0.056	0.147	23,055	39.6	21.8	22.5	50.45	37,000	3.89
C-00-124	0.017	384	3.77	4.07	0.017	0.155	0.057	0.229	35,713	37.9	31.5	23.0	54.36	50,000	7.33
C-00-122	0.019	367	7.79	5.54	0.016	0.165	0.104	0.285	44,797	37.1	26.0	21.3	50.04	30,000	7.44
C-00-157	0.019	385	5.07	4.34	0.008	0.092	0.046	0.146	22,962	38.4	26.5	21.6	51.33	34,000	2.66
C-00-192	0.021	311	6.81	5.11	0.020	0.240	0.072	0.332	52,092	38.5	21.1	21.4	48.79	50,000	11.35
C-00-120	0.021	344	7.89	5.23	0.012	0.141	0.059	0.212	33,316	33.2	29.2	18.1	47.76	45,000	8.18
C-00-148	0.019	350	4.46	3.51	0.013	0.165	0.083	0.261	41,137	40.1	28.0	23.3	54.11	50,000	6.44
C-00-149	0.017	391	3.97	3.67	0.008	0.084	0.043	0.135	21,191	40.3	27.9	23.4	54.26	40,000	3.03
C-00-150	0.023	325	4.35	3.28	0.016	0.122	0.116	0.254	39,806	41.2	18.6	22.3	50.37	37,000	8.89
C-00-188	0.017	367	6.66	4.19	0.009	0.093	0.035	0.137	21,445	32.8	25.2	18.3	45.21	30,000	4.46
C-00-152	0.017	382	4.88	3.41	0.008	0.118	0.047	0.173	27,309	39.0	29.6	22.6	53.87	44,000	4.43
C-00-159	0.016	406	3.85	3.12	0.009	0.106	0.053	0.168	26,436	40.4	30.0	24.0	55.73	50,000	3.88
C-00-153	0.017	398	4.83	3.48	0.016	0.190	0.096	0.302	47,534	41.9	25.7	24.0	54.70	55,000	8.02
C-00-160	0.021	351	5.68	4.12	0.009	0.077	0.062	0.148	23,216	38.9	20.6	21.4	48.90	50,000	5.66
C-00-158	0.019	356	5.48	4.01	0.005	0.049	0.017	0.071	11,091	36.9	28.3	20.9	50.94	37,000	1.26
C-00-162	0.018	374	4.08	3.17	0.023	0.270	0.152	0.445	70,081	43.3	25.9	25.2	56.32	50,000	11.27
C-00-161	0.018	375	5.96	2.62	0.004	0.086	0.032	0.122	19,370	35.8	27.7	20.1	49.51	50,000	2.19
C-00-164	0.020	347	3.75	2.64	0.006	0.105	0.044	0.155	24,547	37.4	28.3	21.6	51.58	50,000	5.40
C-00-163	0.018	360	5.60	3.76	0.027	0.261	0.102	0.390	60,954	40.9	30.8	24.4	56.69	50,000	10.91
C-00-165	0.021	343	4.75	3.72	0.013	0.170	0.076	0.259	40,815	40.1	26.8	23.1	53.44	60,000	8.64
C-00-166	0.019	351	5.83	4.08	0.004	0.082	0.029	0.115	18,243	35.5	27.2	19.9	48.93	55,000	2.55
C-00-167	0.019	364	4.30	3.23	0.010	0.093	0.054	0.157	24,597	40.0	27.6	23.2	53.81	60,000	4.59
C-00-168	0.021	339	4.62	4.04	0.016	0.190	0.091	0.297	46,729	40.7	24.8	23.4	53.04	50,000	9.47
C-00-186	0.017	373	6.49	4.64	0.015	0.138	0.056	0.209	32,629	32.5	24.5	18.0	44.48	30,000	7.90
C-00-176	0.019	348	5.27	4.35	0.019	0.186	0.075	0.280	43,788	39.8	31.1	23.3	55.59	55,500	9.95
C-00-190	0.026	393	5.83	4.56	0.015	0.166	0.075	0.256	40,196	41.1	25.7	23.6	53.89	37,000	7.20
C-00-187	0.022	300	5.20	4.08	0.020	0.195	0.070	0.285	44,525	39.5	20.9	22.0	49.75	43,000	8.36
C-00-197	0.023	291	6.10	4.69	0.016	0.197	0.082	0.295	46,407	41.0	24.2	23.3	52.95	42,000	9.71
C-00-195	0.023	302	7.50	5.28	0.009	0.075	0.067	0.151	23,699	37.1	19.2	20.2	46.40	39.166	5.96
C-00-199	0.024	283	5.70	4.50	0.012	0.098	0.072	0.182	28,486	38.4	20.8	21.3	48.55	35,000	7.35
C-00-200	0.023	291	6.54	4.95	0.020	0.199	0.074	0.293	45,813	39.2	18.7	21.8	48.58	46,666	9.67
C-00-204	0.020	346	4.78	3.90	0.007	0.095	0.046	0.148	23,352	38.3	25.1	21.7	50.65	45,000	5.19
C-00-206	0.022	322	3.94	3.06	0.018	0.222	0.105	0.345	54,321	42.1	26.1	24.5	55.23	55,000	10.89
C-00-173	0.017	392	3.90	2.98	0.005	0.051	0.027	0.083	13,023	38.8	28.2	22.9	53.13	34,000	2.23
C-00-174	0.019	355	4.77	3.40	0.010	0.123	0.062	0.195	30,715	40.5	25.9	23.4	53.43	18,000	5.55
C-00-169	0.021	338	6.03	4.41	0.008	0.064	0.056	0.128	20,064	39.2	19.4	21.4	48.65	45,000	3.12
C-00-171	0.019	354	4.75	3.48	0.010	0.107	0.061	0.178	27,978	41.4	25.6	23.9	54.20	50,000	5.37
C-00-121	0.018	363	6.02	4.52	0.010	0.119	0.078	0.207	32,647	40.0	23.2	22.0	51.65	26,000	7.35
C-00-123	0.016	402	8.30	5.72	0.029	0.277	0.106	0.412	64,360	39.0	27.2	22.8	52.68	70,000	11.99
C-00-170	0.018	373	3.83	2.84	0.019	0.233	0.102	0.354	55,702	34.4	27.8	19.7	48.39	50,000	10.88
C-00-151	0.015	421	3.82	3.07	0.014	0.176	0.087	0.277	43,645	35.2	28.7	20.1	49.61	50,000	8.45
C-00-177	0.016	404	4.64	3.30	0.014	0.144	0.055	0.213	33,341	38.9	21.9	22.3	49.87	34,000	7.79
C-00-178	0.018	368	4.57	3.64	0.014	0.139	0.054	0.207	32,375	36.3	23.4	20.6	47.85	34,000	7.75
C-00-180	0.016	389	5.93	3.86	0.012	0.115	0.045	0.172	26,876	33.9	23.3	18.7	45.16	28,500	5.88
C-00-181	0.017	372	5.22	3.61	0.015	0.143	0.058	0.216	33,756	32.9	24.8	18.4	45.09	29,000	5.25
C-00-182	0.021	328	6.39	4.52	0.013	0.103	0.073	0.189	29,545	38.4	19.16	21.0	47.94	34,166	5.46
C-00-183	0.022	304	5.55	4.20	0.016	0.164	0.072	0.252	39,484	42.4	25.2	24.3	54.90	35,833	8.68
C-00-184	0.020	334	5.88	4.37	0.015	0.167	0.076	0.258	40,518	41.7	24.9	24.0	54.11	37,000	8.42
C-00-185	0.018	364	4.65	3.53	0.015	0.136	0.054	0.205	31,985	37.7	23.4	21.5	49.22	31,000	5.78
C-00-189	0.021	326	5.23	3.69	0.015	0.157	0.074	0.246	38,586	43.0	24.7	24.9	55.47	35,000	6.87
C-00-201	0.024	287	6.57	5.00	0.012	0.101	0.075	0.188	29,452	37.4	19.7	20.6	46.96	35,000	4.19
C-00-220	0.027	268	6.55	4.58	0	0	0	0	0	27.7	21.0	14.1	37.47	0,000	0.59
C-00-203	0.023	314	4.94	3.74	0.016	0.166	0.079	0.261	40,933	37.7	21.5	21.1	48.19	37,000	10.52

[a] M^2/cm^3 determined by ATM sonic sifter.
[b] Mean Particle Size, μ determined by ATM sonic sifter.
[c] Moisture, determined by Azeotropic Distillation and Vacuum Oven (ASTA methods 2.0 and 2.3). Mean value of both methods.
[d] Determined by Beckman Water Activity Hygrometer Hydroline.
[e] % Nordihydrocapsaicin, determined by HPLC (Hoffman et al., 1983)
[f] % Capsaicin, determined by HPLC (Hoffman et al., 1983)
[g] % Dihydrocapsaicin, determined by HPLC (Hoffman et al., 1983)
[h] % Capsaicinoids, determined by HPLC (Hoffman et al., 1983)
[i] Calculated Scoville, calculated using method described by Todd et al. (1977)
[j] Determined using Hunter Colorimeter, Model D25M-9
[k] Scoville, determined using ASTA method 21: Scoville Heat Test.
[l] Sensory Heat, determined using new sensory method.

Table 5—Results of regression analyses on sensory heat vs several analytical measurements of 40 ground red peppers

Variables	r	F	Equation of line
Sensory vs:			
% Capsaicinoids[a] and ΔE[b]	0.959	212.6**	Sensory = 34.18 (% Capsaicoinoids) – 0.216 (ΔE) + 10.24
% Capsaicinoids and b[c]	0.954	188.8**	Sensory = 34.66 (% Capsaicinoids) – 0.364 (b) + 7.07
% Capsaicinoids and a_w[d]	0.946	159.0**	Sensory = 30.38 (% Capsaicinoids) + 0.522 (Aw) – 2.12
% Capsaicinoids and Moisture[e]	0.943	151.1**	Sensory = 31.02 (% Capsaicinoids) + 10.272 (Moisture) – 1.61
% Capsaicinoids	0.939	284.1**	Sensory = 31.26 (% Capsaicinoids) – 0.214
Calaulated Scoville Heat Units[f]	0.938	280.5**	Sensory = 1.99 (Calculated Scoville) – 0.223
% Capsaicin[g]	0.921	214.9**	Sensory = 45.54 (% Capsaicin) + 0.256
% Nordihydrocapsaicin[h] and ΔE	0.899	78.3**	Sensory = 468.32 (% Nordihydrocapsaicin) – 0.149 (ΔE) + 8.39
Scoville Heat Units[1]	0.475	11.1**	Sensory = 1.45 (SHU) + 0.303
Scoville Heat Units vs:			
Calculated Scoville Heat Units	0.476	5.5	Calculated Scoville = 0.683 (SHU) + 4684.67
Sensory	0.475	11.1**	Sensory = 1.45 (SHU) + 0.303

[a] Total capsaicinoids as determined by HPLC (Hoffman et al., 1983).
[b] Change in total color, determined by Hunter Colorimeter, Model D25M-9.
[c] Color value, Hunter Colorimeter, Model D25M-9.
[d] Water activity determined by Beckman Water Activity Hygrometer.
[e] Moisture determined by Azeotropic Distillation.
[f] Calculated using method described by Todd et al. (1977).
[g] Capsaicin as determined by HPLC (Hoffman et al., 1983).
[h] Nordihydrocapsaicin as determined by HPLC (Hoffman et al., 1983).
[i] ASTA method 21.0; Scoville Heat Test.
** Statistically significant at 99% level of confidence.

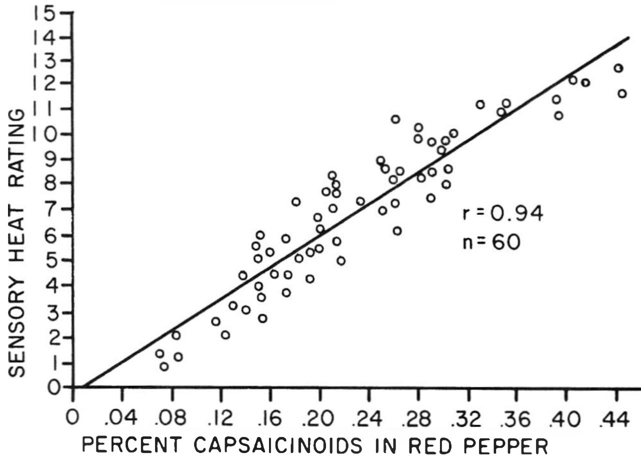

Fig. 2—Sensory heat ratings versus concentration of capsaicinoids in 60 natural ground red peppers.

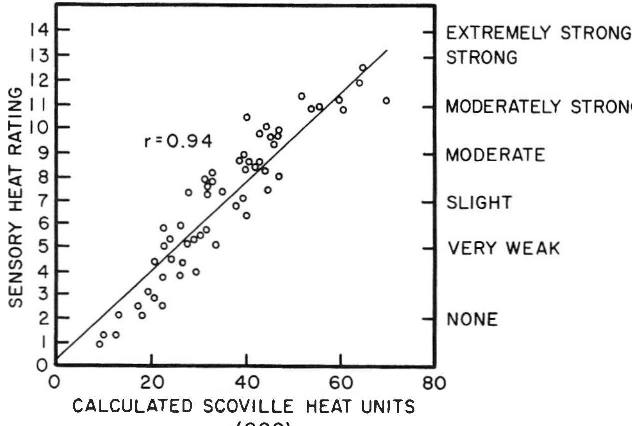

Fig. 3—Sensory heat ratings versus calculated Scoville Heat Units for 60 red peppers. Scoville Heat Units calculated based upon method described by Todd et al. (1977).

plained by variation in the percent capsaicinoids and moisture, and percent capsaicinoids and Aw.

The collaborative study demonstrated the ability of the new method to accurately duplicate results (Table 7). Results of the replicate sample testing within one panel demonstrates the intra-panel accuracy of the method (Table 8).

Table 6—Predictive value of percent capsaicinoids as determinant of sensory heat in 20 red peppers

% Capsaicinoids	Predicted sensory heat rating[a]	Sensory heat rating[b]	Actual-Predicted
0	– 0.21	0.59	0.80
0.083	2.39	2.23	– 0.16
0.128	3.79	3.12	– 0.67
0.172	5.17	5.88	0.71
0.178	5.36	5.37	0.01
0.188	5.67	4.19	– 1.48
0.189	5.70	5.46	– 0.24
0.195	5.89	5.55	– 0.34
0.205	6.20	5.78	– 0.42
0.207	6.26	7.75	1.49
0.207	6.26	7.35	1.09
0.213	6.45	7.79	1.34
0.216	6.54	5.25	– 1.29
0.246	7.48	6.87	– 0.61
0.252	7.67	8.68	1.01
0.258	7.86	8.42	0.56
0.261	7.95	10.52	2.57
0.277	8.45	8.54	0.09
0.354	10.86	10.88	0.02
0.412	12.67	11.99	– 0.68
		M.A.D.[c]	0.78

[a] From linear regression equation, Sensory Heat Rating = 31.27 (% Capsaicoinoids) – 0.21. r = 0.94.
[b] Mean of 10 trained panelists. 1.25 = threshold, 5 = slight, 10 = moderate, 15.0 = strong.
[c] Mean Average Deviation. Average of absolute values of differences between predicted and actual sensory values.

CONCLUSIONS

THIS NEW METHOD avoids the problems inherent in the Scoville method. Heat build up, taste fatigue and increased threshold are accounted for by use of a standardized initial sample, as well as timed rinsing between samples. Ethanol bite is avoided by use of an aqueous extraction. The panel data may be manipulated statistically due to the linearity of

Table 7—Results of collaborative study on red pepper sensory heat method[a]

	Sensory heat ratings							
	Lab #1			Lab #2	Lab #3	Inter-Lab	Inter-Lab	Inter-Lab
	Panel A	Panel B	Panel C	Panel D	Panel E	X	σ	σ/X
Pepper	(n = 10)	(n = 10)	(n = 9)	(n = 9-11)	(n = 4-5)	(n = 5)	(n = 5)	(n = 5)
1	0.6 ± 0.6	0.6 ± 0.6	0.4 ± 0.5	0.5 ± 0.8	1.0 ± 1.1	0.6	0.23	0.38
2	3.0 ± 1.7	4.0 ± 2.3	4.7 ± 2.3	4.8 ± 1.3	6.1 ± 1.4	4.5	1.1	0.22
3	5.4 ± 2.1	5.1 ± 3.1	3.4 ± 2.0	6.0 ± 2.0	6.5 ± 1.4	5.3	1.2	0.22
4	8.4 ± 2.5	9.4 ± 2.1	8.3 ± 1.7	4.4 ± 1.7	7.5 ± 1.4	7.6	1.9	0.25
5	10.0 ± 1.4	7.8 ± 2.4	10.0 ± 1.7	9.7 ± 2.1	11.0 ± 1.4	9.7	1.2	0.12
6	12.6 ± 1.7	9.1 ± 1.7	11.3 ± 0.9	11.9 ± 1.7	12.0 ± 1.4	11.4	1.4	0.12

[a] Means and standard deviations for 6 ground red peppers tested in 3 labs by 5 separate panels. Laboratory Means, Standard Deviations and Coefficients of Variations. Coefficient of Variation = σ/X; an approximation of method inter-laboratory precision.

Table 8—Sensory heat ratings for blind duplicate samples of red pepper[a]

Pepper	Sensory heat rating Session 1	Sensory heat rating Session 2
A	3.3	2.7
B	3.5	4.6
C	5.7	5.9
D	5.9	5.6
E	6.6	4.8
F	8.5	9.8
G	8.5	8.6
H	8.6	8.8
I	10.7	10.9
J	10.9	10.9

[a] Each sample evaluated at 2 different sessions by the same panel (n = 10). No pair of duplicate samples is different when analyzed by a paried t test.

the scale and the number of panelists. Reference standards are included. Extraction time is reduced from 16 hr to 20 min. Reproducibility of results has been demonstrated. The error of central tendency is avoided by not having a "middle" sample. The new method is more comparable to normal food usage as it is an aqueous rather than ethanol extraction.

This method is currently being used for routine laboratory analysis of red pepper heat. Results have been consistent and continue to correlate well with HPLC data. The method has also been used for sensory evaluation of black pepper heat. The American Society for Testing and Materials (ASTM, Committee E-18) has conducted a collaborative study testing the new method in comparison to the Scoville Method. ASTM E-18 is currently preparing to document it as a standardized test method. A modification of the method is also being prepared for oleoresin capsicum.

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Assessment of Freezing Time Prediction Methods

A. C. CLELAND and R. L. EARLE

ABSTRACT

A systematic procedure for testing the accuracy of freezing time prediction methods is discussed. It involves carrying out freezing time predictions by an accurate numerical method as well as the method under test, and careful cross-checking of prediction errors for correlation with a number of physical parameters. Criteria are given for selection of an appropriate numerical method. The need for systematic cross-checking is illustrated by comparisons between four recent prediction methods. The comparisons show the strengths and weaknesses of the four methods. They also indicate general shortcomings in available methods and data, and so highlight those areas in which future research might be most profitable.

INTRODUCTION

TO PRESENT a complete bibliography of work published on methods for freezing time prediction would be a major task, with hundreds of references to be cited. In spite of this considerable amount of research there is still no definitive prediction method that can be used with confidence by food engineers. Worse, the literature often presents a situation of conflict, with prediction methods performing well in some circumstances but not in others.

One of the surprising aspects of the food freezing literature is the paucity of systematically collected experimental data. The present authors have published extensive blocks of data for "Tylose," (a 23% methylcellulose, 77% water gel often used as an analogue in food freezing experiments), plus limited data for meat and potato (Cleland and Earle, 1977a; 1979a, b). Other major contributions to the pool of data have been those of Linge (1973) whose data are for meat and Tylose; de Michelis and Calvelo (1983) whose data are for meat; Hung and Thompson (1983) whose data are for Tylose, meat, potato and fish; and Purwadaria and Heldman (1982) who conducted experiments for meat in anomalous shapes. In all these cases the full set of conditions used in the experiments are listed. In other cases where experimental data have been published, full details have generally not been given. The only sure way to evaluate prediction methods is by comparison with sufficiently precise experimental data. Any definitive freezing time prediction method must be able to stand up to the data, subject of course to the necessary tolerances allowed for imprecise thermal properties and experimental error.

Freezing time calculation methods are often classed into two groups – numerical methods (finite differences and finite elements), and simple formulae. Within the latter group there are methods based on adaptation of analytically derived formulae, and those derived by curve fitting experimental data. Wherever any freezing time prediction method is used some imprecision will be inevitable. This imprecision may arise from one of three sources: (a) uncertainty in thermal data for the material being frozen (k, ρ and c); (b) imprecise knowledge of the freezing conditions, particularly the surface heat transfer coefficient h; and (c) inaccuracy arising from assumptions or approximations made in the derivation of the prediction method. A common approach is to assume that the best freezing time prediction formula will be the one in which error arising from category (c) is least. This is perhaps too simplistic an approach. Food thermal properties vary substantially as the ice fraction of the food changes. Most of the ice is formed across a relatively narrow temperature band below T_f. For most foods at temperatures below −25°C the change in ice content with temperature is small so for practical purposes the food can be considered fully frozen. Some freezing time prediction methods require thermal data across the whole temperature range, and are therefore sensitive to inaccurate knowledge of k and ρc in the narrow temperature range below T_f where these properties change rapidly. The size of the changes has been illustrated by Hseih et al. (1977). In contrast, other methods need k and ρc values for only the parts of the overall temperature range where the change in thermal properties with temperature is small. It is conceivable that such a method, by avoiding the need for thermal data in a temperature range where these data are subject to considerable uncertainty, may be more accurate than one which requires thermal data across the whole temperature range. Therefore complete separation of the effects of prediction method inaccuracy and thermal data uncertainty is not possible.

The net result of these factors is that it is impossible to decide on purely theoretical grounds what freezing time prediction formula is most accurate. Testing must be carried out against experimental data. The major problem in this is that the cause of disagreement between experiment and prediction is a combination of thermal data uncertainty, imprecise measurement and control of the experimental environment, and prediction method inaccuracy. It is inevitable that predictions from an accurate method will not fit low quality experimental data at all well. Many publications in the past have not attempted to quantify the individual components of the total difference between prediction and experiment, and hence the accuracy of the prediction method alone has not been established. This paper proposes a procedure for systematically analyzing the components of observed differences between experimental and predicted freezing times. This procedure is intended to aid the critical examination of freezing time predictions so that reasons can be discerned for inaccuracy or inconsistency when comparing predictions to experimental data. The results should be such as to give some guidance to workers having to make predictions, and to researchers wishing to know the areas in which further information is most needed.

THEORETICAL BASIS

IN ORDER TO ASSESS reasons for the differences between predicted and experimental freezing time a mechanistic view of how process parameters affect freezing time is useful. That used most commonly is Plank's equation. If exprssed in general form it enables the effect of various potential causes of poor agreement to be postulated:

$$(\text{Freezing time}) = \frac{(\text{product enthalpy change})}{(\text{temp. driving force})}\left[(\text{constant})\frac{D}{h} + (\text{constant})\frac{D^2}{k_s}\right] \quad (1)$$

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Probably the most difficult factor to measure, and therefore the major error source is the surface heat transfer coefficient, h. Problems arise experimentally in ensuring that it is the same on all surfaces, that it does not vary with time, and in measuring it accurately. Error in it may be either systematic or random. Errors in h are most noticeable at low values of the Biot number. When Bi is small, only the first term within the rightmost bracket in Eq. (1) is significant. Thus the freezing time is only slightly affected by thermal conductivity. A systematic error in h will affect the mean difference between prediction and experimental result, whereas random (experimental) error will affect the spread of results at low Bi.

The second most important source of disagreement is probably thermal data. Errors in specific heats (above or below the freezing region), and the enthalpy change during freezing will affect the mean difference between experiment and prediction more than the spread. Further, if the mean is affected the extent of the effect is likely to be largely independent of the Biot number.

Errors in thermal conductivity will be of little consequence at low Biot numbers, and most significant at high Biot numbers where only the second term within the rightmost bracket of Eq. (1) is important. The mean difference at high Bi will be affected if k is incorrect, but not the spread.

Another factor can cause a systematic underprediction of freezing time for high Biot numbers. It has been observed that at high Biot numbers predictions by finite differences underpredicted freezing times, and also the shapes of the predicted and measured temperature/time profiles were very different (Cleland et al., 1982). However, at lower Biot numbers (0.2–4) the shapes of experimentally measured and predicted temperature profiles were virtually identical. In these circumstances a systematic error in thermal conductivity could explain the observed differences in freezing time, although it would have to be 25% in magnitude to do so, and this is unlikely. However, a systematic error would not explain good prediction of the shape of internal temperature profiles at low Bi, but not at high Bi, as was observed. The conclusion reached was that the formation of ice during freezing was not necessarily in equilibrium with the removal of heat, so that significant supercooling could persist right throughout the freezing process. Measurements of thermal conductivity, enthalpy and/or ice fraction are usually taken under equilibrium conditions. Use of thermal conductivity and specific heat obtained from steady state data in freezing calculations implies an assumption that the shapes of these curves are independent of rate. This is not necessarily true. The hypothesis of significant supercooling at high freezing rates was tested (Cleland et al., 1982). The shapes of both the enthalpy/temperature and the thermal conductivity/temperature curves were adjusted so that ice formation was assumed to occur at lower temperatures than was the case in equilibrium data. Use of the adjusted thermal properties led to better predictions of freezing time, and of the shape of the temperature/time curves at high Bi (high freezing rate), but as expected to poorer predictions at low Bi than were achieved with thermal properties derived from equilibrium data.

A wide spread of results occurring only at high Bi suggests poor control of experimental parameters other than the surface heat transfer coefficient.

Temperatures are normally minor error sources as they can be measured with relative precision compared to heat transfer coefficients and thermal properties. Three temperatures are of importance – the initial temperature of the material, the temperature at which it starts to freeze, and the cooling medium temperature. Imprecise evaluation should not be a major problem. However, imprecise control of temperature, particularly that of the cooling medium can be critical. Poor control of cooling medium temperature will lead to a large spread of results where the cooling medium and mean product temperatures come close together, that is, where the final product temperature at the end of freezing is only slightly higher than the cooling medium temperature.

The final important source of disagreement between predictions and experimental results is experimental technique associated with test object construction. Where a freezing time is measured, thermocouples or other temperature measuring devices must be placed in the object, and the object must be constructed to be as homogenous as possible (if homogeneity is required). If experiments are conducted for only one or two dimensional heat transfer insulation of other edges of the test object will never be 100% successful. The importance of "edge" heat transfer can vary substantially depending on the experimental apparatus.

Taking into account all these things a check list has been constructed in Table 1 which enables the most probable sources of difference between experiment and prediction, other than prediction method, to be assessed.

PREDICTION METHOD ASSESSMENT PROCEDURE

A TEST PROCEDURE for assessing freezing time prediction formulae can be proposed that uses the theoretical basis just discussed.

(1) Collect experimental data in which the following parameters are varied across wide ranges: (a) Biot number, (b) factors affecting the total product enthalpy change during freezing, and the ratios of latent to sensible heat components within it. These are the block initial temperature and the temperature at which freezing is deemed complete, (c) the cooling medium temperature, (d) the material being frozen. This varies the relativity of frozen phase and unfrozen phase thermal properties.

(2) Add previously published experimental data to the pool of data to be used unless insufficient information is given to be sure of its reliability. A major problem here is that it is often difficult to obtain thermal properties to go with data sets used by other workers. Satisfactory properties can be estimated from compositional factors for many foods (Comini et al., 1974; Heldman, 1982) if published data are not available.

Table 1—Identification of likely causes (other than prediction method inaccuracy) of disagreement between predicted and experimentally measured freezing times

Observation	Most likely causes
Spread of results only at low Bi	Poor control or measurement of the surface heat transfer coefficient
Offset mean only at low Bi	Systematic error in surface heat transfer coefficient
Offset mean only at high Bi	Error in thermal conductivity
	Freezing rate influence on thermal data
Spread of results only at high Bi	Poor experimental technique other than for the surface heat transfer coefficient
Offset mean at all Bi	Error in enthalpy/specific heat
	Systematic experimental error
Spread of results at all Bi	Poor experimental technique
Spread of results at relatively high cooling medium temperature	Poor control of cooling medium temperature

(3) Carry out predictions for a numerical freezing time prediction method (finite differences or finite elements). It is not possible to completely remove prediction method uncertainty, but the numerical methods are the closest to an "exact" freezing time prediction method that exists. If they are used carefully the residual difference between experimental results and the prediction is that due to thermal data, and experimental error only. For any freezing material the same thermal data are used in all numerical calculations so the effect of thermal data error on the differences between experimental and predicted freezing times will be systematic. Reference to Table 1 suggests that if the mean prediction error using finite differences or finite elements deviates from zero a systematic error in thermal data is the most likely cause. The spread of prediction errors reflects mainly the experimental error. It can be affected by error in thermal conductivity but this latter should be correlated with Biot number and is therefore detectable. The importance of carrying out the numerical calculations is that they establish a baseline to which to compare other prediction formulae. Digital computers are widely available, and a variety of finite difference and finite element programmes have existed for a number of years so use of this type of freezing time calculation should not be a problem.

(4) Predictions from other freezing time prediction formulae can now be examined. In general it would be surprising if any such method performs significantly better than a numerical method. However, as discussed previously many simple methods do not need all the k and ρc data necessary for finite differences or finite elements. If the data not used (especially those across the main phase transition zone) are a significant source of error, it is possible that the simple method, by avoiding these data, could do better than a numerical method, but the improvement is unlikely to be major. A further important consideration is that if experimental error is the main cause of disagreement between experiment and prediction the prediction errors from an accurate simple prediction formula and finite differences or finite elements would be expected to be highly correlated. This requires only a simple statistical test. Thus a reliable simple prediction formula should agree with both experimental data and numerical predictions.

(5) If, at the completion of the study a new freezing time prediction method or a refinement of an existing one is proposed it is helpful to publish both a worked example calculation, and detailed information on thermal properties used. This information ensures that other workers do not unwittingly misinterpret the new technique. Journals are conscious of the need to keep papers as brief as possible, but extensive experience with trying to use methods published by other workers indicates worked examples have an important role, and are therefore well worthwhile to include.

Before illustrating the use of this procedure it is first necessary to discuss finite difference schemes as the procedure assumes that a finite difference or finite element scheme with almost zero error exists. All finite difference schemes are not equivalent, and in particular some systematic errors can build up, so it is important that the most accurate is used.

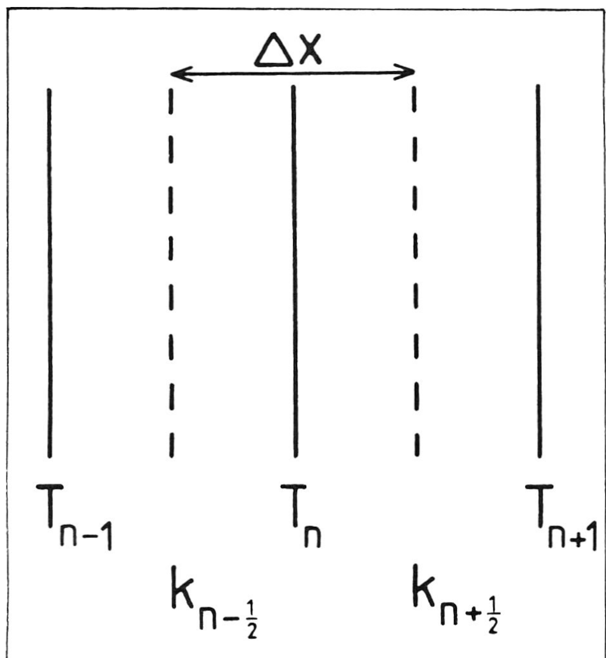

Fig. 1—Schematic illustration of a small element of thickness Δx within an infinite slab of cross-sectional area A.

FINITE DIFFERENCE METHODS

VIRTUALLY ALL THE MAJOR BLOCKS of experimental data are for regular shapes and homogeneous materials. In these cases finite elements have no advantages over finite differences, so only the latter are discussed although similar principles could be applied to both types of method. To study the degree of precision achievable with a finite difference method it is first necessary to re-establish the basis of the method. The slab case is shown in Fig. 1, but any other shape could have been studied. A heat balance over a small section of the slab gives:

$$\left(Mc\frac{\partial T}{\partial t}\right)_n = \left(kA\frac{\partial T}{\partial x}\right)_{n+½} - \left(kA\frac{\partial T}{\partial x}\right)_{n-½} \qquad (2)$$

This equation assumes that the material within the nth element has mass M, volume $A\Delta x$, specific heat c, and can be represented by a unique temperature T_n. The specific heat capacity for this region is evaluated at T_n. The conduction heat flows are evaluated at positions (n+½) and (n−½).

The general heat flow equation is derived from Eq. (2) by setting $M = A\Delta x\rho$ and rearranging:

$$\left(\rho c\frac{\partial T}{\partial t}\right)_n = \frac{1}{\Delta x}\left[\left(k\frac{\partial T}{\partial x}\right)_{n+½} - \left(k\frac{\partial T}{\partial x}\right)_{n-½}\right] \qquad (3)$$

which in the limit as $\Delta x \to 0$ becomes:

$$\rho c\frac{\partial T}{\partial t} = \frac{\partial}{\partial x}\left(k\frac{\partial T}{\partial x}\right) \qquad (4)$$

Often Eq. (4) is written as:

$$\frac{\partial T}{\partial t} = \alpha\frac{\partial^2 T}{\partial x^2} \qquad (5)$$

This is only true if k is independent of position in the material, that is, the material is homogeneous. Further, if temperature varies with position and k with temperature, k cannot be removed from the bracket in Eq. (4), and so Eq. (5) is invalid. Another alternative is:

$$\frac{\partial T}{\partial t} = \frac{\partial}{\partial x}\left(\alpha\frac{\partial T}{\partial x}\right) \qquad (6)$$

For α to be transferred into the differential term on the right hand side, yet Eq. (2) to be still correct, ρc must not vary with position (i.e. $\rho c_{n+½} = \rho c_n = \rho c_{n-½}$). Temperature may vary with position so the implication is that Eq. (6) only applies if ρc is not temperature-variant, although k may vary.

A variety of difference schemes exist. Some of the most common are shown in Table 2. It is not important at this juncture to list which authors have used which method, but

Table 2—Some finite difference schemes commonly used in freezing time calculations. All schemes are illustrated for the infinite slab case

(A) Lees scheme

$$(\rho c)_n^i \frac{T_n^{i+1} - T_n^{i-1}}{2\Delta t} = \frac{1}{3(\Delta x)^2} \Big\{k_{n+1/2}^i \Big[\Big(T_{n+1}^{i+1} - T_n^{i+1}\Big) + \Big(T_{n+1}^i - T_n^i\Big) + \Big(T_{n+1}^{i-1} - T_n^{i-1}\Big)\Big] - k_{n-1/2}^i \Big[\Big(T_n^{i+1} - T_{n-1}^{i+1}\Big) + \Big(T_n^i - T_{n-1}^i\Big) + \Big(T_n^{i-1} - T_{n-1}^{i-1}\Big)\Big]\Big\}$$

(B) Modified Crank-Nicholson scheme

$$(\rho c)_n^i \frac{T_n^{i+1} - T_n^i}{\Delta t} = \frac{1}{2(\Delta x)^2} \Big\{k_{n+1/2}^i \Big[\Big(T_{n+1}^{i+1} - T_n^{i+1}\Big) + \Big(T_{n+1}^i - T_n^i\Big)\Big] - k_{n-1/2}^i \Big[\Big(T_n^{i+1} - T_{n-1}^{i+1}\Big) + \Big(T_n^i - T_{n-1}^i\Big)\Big]\Big\}$$

(C) Fully Implicit scheme

$$(\rho c)_n^i \frac{T_n^{i+1} - T_n^i}{\Delta t} = \frac{1}{(\Delta x)^2} \Big[k_{n+1/2}^i \quad T_{n+1}^{i+1} - T_n^{i+1}\Big) - k_{n-1/2}^i \Big(T_n^{i+1} - T_{n-1}^{i+1}\Big)\Big]$$

(D) Fully Explicit scheme

$$(\rho c)_n^i \frac{T_n^{i+1} - T_n^i}{\Delta t} = \frac{1}{(\Delta x)^2} \Big[k_{n+1/2}^i \Big(T_{n+1}^i - T_n^i\Big) - k_{n-1/2}^i \Big(T_n^i - T_{n-1}^i\Big)\Big]$$

(E) Enthalpy Transformation (Explicit) scheme

$$\frac{H_n^{i+1} - H_n^i}{\Delta t} = \frac{1}{2(\Delta x)^2} \Big[k_{n+1/2}^i \Big(T_{n+1}^i - T_n^i\Big) - k_{n-1/2}^i \Big(T_n^i - T_{n-1}^i\Big)\Big]$$

where H_n^{i+1} and T_n^{i+1} are related after each time step.

(F) Modified Crank-Nicholson scheme using thermal diffusivity

$$\frac{T_n^{i+1} - T_n^i}{\Delta t} = \frac{1}{2(\Delta x)^2} \quad \frac{k}{\rho c}\Big)_{n+1/2}^i \Big[\Big(T_{n+1}^{i+1} - T_n^{i+1}\Big) + \Big(T_{n+1}^i - T_n^i\Big)\Big] - \Big(\frac{k}{\rho c}\Big)_{n-1/2}^i \Big[\Big(T_n^{i+1} - T_{n-1}^{i+1}\Big) + \Big(T_n^i - T_{n-1}^i\Big)\Big]\Big\}$$

rather to understand the relative merits of each scheme. If run against an analytical solution for constant thermal properties such as those reported by Carslaw and Jaeger (1959) all these schemes would give results that agreed with the analytical solution. However, where thermal properties change (for example in freezing calculations in which latent heat release is treated as a temperature dependent specific heat) some differences in process time prediction will result in spite of all input data being identical. The reasons for these differences can be established from the previous discussion. Scheme F, and any other scheme based on Eq. (5) or Eq. (6) rather than Eq. (2) and (4) are incorrect if both ρc and k vary with temperature.

Within schemes A to E differences arise from two causes. Firstly the truncation errors arising from Scheme A are smaller than those for the other schemes (due to the better approximation of $\partial T/\partial t$ on the left hand side). Secondly, the time level at which thermal properties are evaluated is important. Ideally on the left hand side position is held constant at the mid-point level (n) and the thermal properties evaluated at the mid-point time level. Only Schemes A and E have this property. On the right hand side the derivatives with respect to space and the thermal properties should be evaluated at the mid-point time level for the difference scheme. This is done for Scheme A, but not for any of the others. Systematic error can result. Consider Scheme B used in a freezing time prediction. Ideally k should be evaluated at $T^{i+1/2}$, and not T^i (this can be done by iterative calculation). During freezing as temperature decreases thermal conductivity always increases. Therefore a thermal conductivity evaluated at T^i will always be slightly lower than the correct value at $T^{i+1/2}$ (a lower temperature). Hence use of T^i for thermal conductivity evaluation will lead to a systematic underestimation of k during freezing, consequential underprediction of heat conduction rate, and overestimation of the freezing time. This error is reduced as the time step is decreased as T^i and $T^{i+1/2}$ differ by less. Scheme A will therefore be most accurate, but the results for the other schemes except Scheme F will converge towards Scheme A as Δt is reduced. Scheme A was first used for freezing time prediction by Bonacina and Comini (1971), who realized the potential problems with the other schemes.

Table 3—Approximate thermal data used for calculation of freezing times of strawberry slabs

Temperature (°C)	k (W/m°C)	Temperature (°C)	ρc (J/m^3°C x 10^{-6})
−40.0	2.08	−40.0	2.15
−5.0	2.08	−5.0	2.15
−3.0	1.74	−0.8	134.3
−1.5	1.08	−0.5	4.09
−0.8	0.65	40.0	4.09
−0.5	0.54		
40.0	0.54		

To illustrate the effects just discussed calculations were carried out for an example analogous to that presented by Heldman (1983). His example was freezing of a 2 cm diameter spherical strawberry. The example used here was the slab equivalent (to match Table 2) with thermal properties represented by the data in Table 3. These are approximate values based on the triangulation method of Comini et al. (1974). Schemes A and E were used with thermal properties calculated by linear interpolation of Table 3. The sphere equivalent of Scheme A predicted a freezing time of 11.55 min which is within the range of times calculated by Heldman (1983) for the strawberry sphere, and therefore indicates that the approximate thermal properties were satisfactory.

The very rapid change in apparent volumetric specific heat capacity presents a problem for all schemes except Scheme E. This problem is "jumping" of the latent heat peak. It has been discussed elsewhere (Cleland and Earle, 1977b), and can be detected by building a heat balance into the finite difference program. In fact, a heat balance should be regarded as a standard item within any finite difference program as it is an excellent way to detect problems in finite difference calculations or scheme formulation. Scheme A is more sensitive than the other schemes to jumping, but

in all cases reduction of the time step will remove the problem. The calculation results in Table 4 are affected by this problem, but still show the expected trend with Schemes B to E only approaching the Scheme A results at small time step sizes.

The effects arising from using a finite difference scheme in which all thermal property evaluations are not centrally located may not be large, but if a finite difference scheme is to be used as the best approximation to an "exact" freezing time prediction method such effects must be eliminated because the errors introduced are systematic. Further, accurate prediction of analytical solution results for constant thermal properties does not mean a scheme is accurate if thermal properties change.

APPLICATION OF THE EVALUATION PROCEDURE

TO DEMONSTRATE the evaluation procedure, three sets of data and four prediction methods were chosen for the comparison. The data used were those of the current authors (Cleland and Earle, 1977a; 1979a, b), those of de Michelis and Calvelo (1983), and those of Hung and Thompson (1983). Each set of authors has given details on experimental apparatus and measurement systems. Prediction methods were those of Pham (1984a, b), Hung and Thompson (1983), de Michelis and Calvelo (1983), and in the case of the present authors, the prediction method used was a recent version, Cleland and Earle (1982).

The first step in the analysis was to examine the data sets. That of Cleland and Earle covers a wide range of Bi, T_a and T_i but was limited to only one final temperature, and made little use of materials other than Tylose. That of Hung and Thompson has wide variation of all parameters, covers several materials, but is restricted to one final temperature. The third set, de Michelis and Calvelo, has some variation of final product temperature, but all data are for one material only, and the variation of T_i in particular is more limited. Amalgamation of the three sets of data provides a data base of 275 experiments for five materials, with two final temperatures, a variety of regular geometries, and wide variation of Bi, T_i and T_a.

The second step was to carry out finite difference calculations by Scheme A, or its multidimensional equivalents, for all sets of data to assess the accuracy of each data set. Thermal data used are shown in Table 5. The choice of thermal properties is a critical step, and other workers may disagree with the values used. However, provided cognizance is taken of the limitations of the data, useful interpretations can still be made. The calculated finite difference results are shown in Table 6. In some cases these differ slightly from previously published results as the thermal data in Table 5 do not exactly match those used previously.

Table 4—Freezing times calculated for a 0.02m thick slab of strawberry material frozen from an initial temperature of 10°C to a final temperature of −25°C, in a cooling medium at −35°C with a surface heat transfer coefficient of 70 W/m²°C. Space step Δx = 0.001m

Time step (s)	Freezing times (min) Scheme A	Scheme B	Scheme C	Scheme D	Scheme E
12.0	–[a]	–[a]	–[a]	–[b]	–[b]
6.0	–[a]	35.82	35.76	–[b]	–[b]
3.0	–[a]	35.75	35.73	–[b]	–[b]
1.5	35.57[a]	35.70	35.71	–[b]	–[b]
0.75	35.63	35.68	35.68	–[b]	–[b]
0.50	35.64	35.67	35.67	35.67	35.65
0.25	35.64	35.64	35.65	35.66	35.65

[a] Time step too large to prevent jumping of the latent heat peak with this scheme. Heat balance disagreed by more than 0.25%.
[b] No calculation possible due to violation of stability criteria.

The mean for all Tylose experiments is close to zero indicating that the overall enthalpy change was accurate. At high Bi there was consistent underprediction – as discussed previously this could be due to an error in frozen Tylose thermal conductivity, or to freezing rate effects on the thermal properties, or to a combination of these.

For lean beef there was over-prediction of freezing time at high Bi indicating that there could be a consistent underestimation of thermal conductivity of the frozen material in Table 5. At low Bi the mean was close to zero so the enthalpy change was probably not a major source of uncertainty.

The potato data had a mean close to zero, and there was no correlation of prediction error with Biot number.

For carp the small size of the data set means that it is more difficult to draw firm conclusions. The mean prediction error was sufficiently close to zero to suggest that error in the enthalpy change was small, and the prediction error was not dependent on Biot number.

Similarly, for ground beef the data set size was a limiting factor. The mean of +6.6% is sufficiently far from zero to suggest that the total enthalpy change may have been overestimated. There was no trend of results with Biot number.

Taking into account these factors it is possible to comment on the size of the experimental error in each data set. In finite difference predictions the data of Cleland and Earle show a smaller spread (mean standard deviation of 5.1%) than those of de Michelis and Calvelo (mean s.d. of 6.5%), which in turn are less spread than those of Hung and Thompson (mean s.d. of 10.4%). This probably reflects the greater use of plate and liquid immersion freezing by the first two sets of authors, and the reliance on air blast freezing by Hung and Thompson. In particular the choice of cooling medium affects the ability to control the uniformity of surface heat transfer coefficient across a surface. A further factor is the material being frozen. Tylose and potato are more easily packed uniformly in a mold than meat or fish, and this is reflected in the spread of results too. There is no indication that any of the sets of data contain gross discrepancies.

The third stage in the assessment procedure was to carry out calculations for the prediction formulae. For these calculations, thermal data for fully frozen and unfrozen products are required. Those used are consistent with Table 5 and are listed in Table 7. Authors proposing a prediction method need to define how thermal properties are evaluated for their method. Where properties for the "fully" frozen material were required those taken were values in the range −25°C to −40°C. Over this temperature range the food may be considered fully frozen for practical purposes as the ice fraction changes only slightly with temperature, and hence the thermal properties are close to constant. For the method of de Michelis and Calvelo, data were required at the mean of T_f and T_a. These were found by linear interpolation of Table 5. The thermal properties used for the simple formulae are thus consistent with those used for the finite difference calculations. The methods of Table 1 ensure major thermal data errors are detected. The calculated freezing times are summarized in Table 6 for each set of experiments. Some of these times differ from previously published calculations because of the differences in thermal data used.

In analyzing these results it is useful to first review the methods. All methods take into account sensible heat effects, but only those of Pham and de Michelis and Calvelo take account of different final temperature. The method of Cleland and Earle was derived by curve fitting data for −10°C. Similarly Hung and Thompson fitted −18°C data. For these methods approximations for different final temperature can be derived by using values of the enthalpy change due to freezing defined as:

$$\Delta H = L + C_s (T_f - T_{fin}) \quad (7)$$

for Cleland and Earle, and

$$\Delta H = L + C_s (T_f - T_{fin}) + C_L (T_i - T_f) \quad (8)$$

for Hung and Thompson, but these will lead to loss of accuracy. The methods of Pham and de Michelis and Calvelo are very similar except for the manner in which multi-dimensional geometry is taken into account. de Michelis and Calvelo use geometric factors first proposed by Plank. These have been shown to be subject to some doubt (Cleland and Earle, 1979b), so Pham uses an alternative geometry description based on curve fitting of the 72 Tylose brick experiments of Cleland and Earle. Hung and Thompson only studied the slab shape. However, their method can be extended directly to other shapes by use of the EHTD concept of Cleland and Earle (1982). This concept and the mean conducting path used by Pham (1984b) allow all formulae for predicting the freezing time of a slab to be extended to any other regular or irregular shape by defining a single geometric parameter. Table 8 summarizes the performance of the four prediction methods in general terms.

The criteria proposed earlier for assessment of freezing time prediction procedures are: fit to experimental data, fit to finite difference predictions, and fit to experimental error in the case of parameters derived empirically from experimental data. Further, in comparing the four methods the possible inadequacies in the thermal data must be taken into account. The method of Pham gave the most consistent

Table 5—Thermal properties used in finite difference calculations by Scheme A. Linear interpolation was used in all cases (Data sources are listed as footnotes)

Temperature (°C)	k (W/m°C)	Temperature (°C)	ρc (J/m^3°C x 10^{-6})
Tylose			
−40	1.67[a]	−40	1.89[b]
−20	1.66	−16	2.01
−10	1.63	−10	3.52
−6	1.57	−7	5.94
−4	1.47	−5	10.6
−2	1.20	−3	25.3
−1	0.83	−2	44.8
−0.6	0.49	−1	101.0
40	0.61	−0.8	178.0
		−0.7	178.0
		−0.6	10.0
		−0.4	3.71
		40	3.71
Lean beef			
−40	1.58[ac]	−40	1.89[d]
−24	1.53	−30	1.91
−12	1.48	−25	2.02
−8	1.40	−20	2.70
−4	1.28	−15	3.58
−2	0.98	−10	4.55
−1	0.48	−8	5.26
40	0.49	−6	7.71
		−5	10.4
		−4	15.7
		−3	31.2
		−2	53.1
		−1.5	73.7
		−1.3	255.0
		−1.2	255.0
		−1.0	3.66
		40	3.66
Potato			
−40	1.90[a]	−40	1.95[ae]
−20	1.87	−25	2.0
−10	1.82	−15	2.6
−6	1.75	−10	3.4
−4	1.64	−8	4.2
−2	1.45	−6	5.7
−1	1.25	−5	8.7
−0.7	0.75	−4	13.7
−0.6	0.53	−3	19.6
40	0.53	−2	27.1
		−1.5	58.6
		−1.0	101
		−0.8	422
		−0.7	422
		−0.6	3.66
		40	3.66
Ground beef			
−40	1.45[a]	−40	1.95[af]
−24	1.43	−30	1.95
−12	1.41	−25	2.05
−8	1.35	−20	2.6
−4	1.24	−15	3.3
−2	0.90	−10	4.1
−1.2	0.44	−8	5.1
40	0.44	−6	7.2
		−5	9.7
		−4	14.5
		−3	28.0
		−2	49.0
		−1.7	78.0
		−1.5	235
		−1.3	235
		−1.2	3.38
		40	3.38
Carp			
−40	1.66[a]	−40	2.1[af]
−24	1.65	−30	2.2
−12	1.61	−20	2.3
−8	1.56	−15	3.2
−4	1.44	−10	4.3
−2	1.14	−8	5.5
−1	0.87	−6	7.0
−0.8	0.48	−5	10.2
40	0.48	−4	14.0
		−3	31.1
		−2	43.7
		−1.5	100
		−1.2	188
		−1.0	188
		−0.8	3.70
		40	3.70

[a] Calculated from compositional factors by method of Comini et al. (1974)
[b] Derived from Riedel (1960a)
[c] Calculated using data from Morley (1972)
[d] Derived from Riedel (1957)
[e] Derived from Riedel (1960b)
[f] Calculated using data from Hung and Thompson (1983)

Table 6—Summary of comparisons between experimental and predicted freezing times for five prediction methods. All data expressed as percentage differences from experimental data

	METHODS									
	Finite differences		Cleland and Earle		Pham		Hung and Thompson		de Michelis and Calvelo	
Data source	Mean	s.d.	Mean	s.d.	Mean	s.d.	Mean	s.d.	Mean	s.d.
Hung and Thompson										
23 Tylose slabs	−1.3	8.7	−12.7	6.0	−3.8	7.7	1.1	2.9	−4.3	14.1
9 potato slabs	−3.9	4.9	−14.5	4.2	−5.1	3.5	2.9	4.0	−4.2	8.4
9 carp slabs	3.1	12.9	−12.6	9.0	−2.4	11.1	2.8	4.1	6.1	16.9
9 ground beef slabs	6.6	16.6	−11.8	12.7	−1.8	13.9	2.5	4.8	10.1	18.9
9 lean beef slabs	2.8	11.6	−14.7	6.4	−6.0	8.9	0.8	4.0	7.9	14.7
Cleland and Earle										
43 Tylose slabs	0.0	5.3	1.6	2.8	3.9	4.4	23.1	11.5	−4.1	6.2
6 potato slabs	−0.5	5.1	−0.5	1.8	3.7	2.3	21.9	6.5	−2.6	3.5
6 lean beef slabs	4.8	4.7	2.1	4.0	4.1	4.6	23.8	12.6	5.6	4.4
30 Tylose cylinders	−1.8	5.2	−1.0	3.6	0.8	5.1	15.4	9.7	−6.5	8.2
30 Tylose spheres	−0.3	3.3	1.6	5.2	2.3	5.1	15.8	9.6	−0.5	10.3
72 Tylose bricks	−3.8	5.8	−0.9	5.7	−1.3	4.7	12.5	10.5	−26.1	9.6
de Michelis and Calvelo										
5 lean beef slabs	−0.1	7.4	0.9	1.9	−0.9	4.9	1.8	9.2	−2.2	10.5
24 lean beef bricks, rods, finite cylinders	8.6	6.3	6.1	10.3	4.1	6.6	11.8	8.3	−11.3	10.8

results over the whole data set, but for the −10°C data it was matched by Cleland and Earle, and for the −18°C data by Hung and Thompson. The method of de Michelis and Calvelo apparently performed worse in terms of spread than the others. Close examination of the calculations showed that this was at least in part due to the way thermal properties at the average of T_f and T_a were taken. Their prediction method is very similar to that of Pham. The exception is in precooling where a mean of the frozen and unfrozen thermal conductivities is used in both methods, but Pham uses the unfrozen specific heat whereas de Michelis and Calvelo use a mean thermal diffusivity which implies some sort of averaging of the specific heat.

The method of Cleland and Earle performed satisfactorily, both in comparison to finite differences and in comparison to experimental data, for all −10°C data. For the −18°C data it performed better if the cooling medium temperature was below −25°C (mean prediction error of −9.0% compared to −15.1% for cooling medium temperatures above −25°C). This means that the approximation suggested in Eq. (7) is reasonable provided the final center temperature is well below the final product temperature, and of course the further the final center temperature is from −10°C the worse the approximation is. The accuracy of the prediction method cannot be easily separated from the thermal data and experimental error. The comparison of differences with finite difference predictions suggests that the error in this prediction method is about ±10% if the final center temperature is −10°C. At −18°C it performs worse, by perhaps as much as 30% in extreme situations. It is possible that the method has fitted some of the experimental error in the Cleland and Earle data set as it was derived from these data. However, for the only independent −10°C data (de Michelis and Calvelo, lean beef bricks) it performed very consistently compared to finite differences and the method of Pham so it was considered that the extent to which the curve-fitted parameters fitted experimental error was negligible.

The method of Pham predicts equally well at the two final center temperatures, and a comparison of it with the finite difference results suggests that it has an inaccuracy of about ±10%. Like all the other methods the correlation coefficient indicated that it failed to give totally consistent predictions compared to the numerical method. For slabs, cylinders and spheres it contains no parameters derived from curve fitting of experimental freezing data. For these shapes it gave results highly correlated with the finite difference predictions indicating that the physical basis of the method for the three basic shapes is probably sound. For brick shapes the method contains curve-fitted parameters derived from the data of Cleland and Earle. For the independent data of de Michelis and Calvelo it performed well compared to finite differences (r=0.799) so the amount of curve fitting of experimental error in the Cleland and Earle data is probably small.

Table 7—Thermal data used for calculation of freezing times by the four simple methods under study

Property	Tylose	Potato	Lean beef	Ground beef	Carp
k_L (W/m°C)	0.55	0.53	0.50	0.44	0.48
k_s (W/m°C)	1.65	1.90	1.55	1.45	1.65
C_L (J/m^3°C x 10^{-6})	3.71	3.66	3.65	3.38	3.70
C_s (J/m^3°C x 10^{-6})	1.90	1.95	1.90	1.95	2.10
L (J/m^3 x 10^{-6})	209	235	209	188	218
T_f (°C)	−0.6	−0.6	−1.0	−1.2	−0.8

As expected, the Hung and Thompson method showed reverse trends to Cleland and Earle. It performed satisfactorily at −18°C, but not at −10°C. The poor fit to −10°C final center temperature data was general, and not affected by the cooling medium temperature, Biot number or initial temperature. Testing of the method to see if it contained any consistent fitting of experimental error was limited as the only completely independent data for freezing to −18°C were five lean beef slab runs of de Michelis and Calvelo. Table 6 shows that for these it produced results largely consistent with both finite differences and the method of Pham, but the spread of results was somewhat greater. The Hung and Thompson formula may therefore contain a small amount of experimental error-fitting to fit the Hung and Thompson data so well in spite of the large spread of finite difference results for the data set.

Tables 6 and 8 suggest that the method of de Michelis and Calvelo was less reliable. As has been discussed the method is particularly sensitive to thermal property estimations – in their paper de Michelis and Calvelo chose different thermal properties. For the basic shapes (slabs, cylinders

Table 8—Summary of percentage differences between (A) experimental data and predicted freezing times, and (B) predicted freezing times from the methods under test and predicted freezing times from finite differences

Data source	Methods			
	Cleland and Earle	Pham	Hung and Thompson	de Michelis and Calvelo
All 275 experiments; A — comparison to experimental data				
Mean	−2.4	0.3	12.5	−8.7
Standard deviation	8.5	6.7	11.8	15.1
Maximum	23	18	44	38
Minimum	−30	−24	−17	−48
Range enclosing 90% of data	−19 to 9	−10 to 9	−4 to 31	−35 to 15
Correlation (r) with finite differences	0.439	0.762	0.354	0.619
207 experiments to −10°C; A — comparison to experimental data				
Mean	0.5	1.3	15.9	−11.8
Standard deviation	6.0	5.4	11.4	13.9
Maximum	23	15	44	16
Minimum	−28	−13	−17	−48
Range enclosing 90% of data	−9 to 10	−9 to 8	−3 to 34	−36 to 8
Correlation (r) with finite differences	0.665	0.738	0.557	0.501
68 experiments to −18°C; A — comparison to experimental data				
Mean	−10.9	−2.8	2.3	0.8
Standard deviation	9.3	9.2	6.0	14.9
Maximum	16	18	30	38
Minimum	−30	−24	−6	−28
Range enclosing 90% of data	−24 to 4	−17 to 13	−5 to 9	−17 to 28
Correlation (r) with finite differences	0.608	0.929	0.552	0.857
All 275 experiments; B — comparison to finite differences				
Mean	−2.1	0.5	12.8	−8.4
Standard deviation	8.6	5.1	11.6	12.0
Maximum	18	14	43	15
Minimum	−34	−15	−24	−50
Range enclosing 90% of data	−21 to 8	−9 to 8	−9 to 28	−30 to 9
207 experiments to −10°C; B — comparison to finite differences				
Mean	1.3	2.1	16.7	−11.0
Standard deviation	5.0	4.3	9.4	12.1
Maximum	18	14	43	15
Minimum	−21	−14	−14	−49
Range enclosing 90% of data	−7 to 9	−5 to 8	−1 to 31	−31 to 6
68 experiments to −18°C; B — comparison to finite differences				
Mean	−12.4	−4.4	0.8	−0.8
Standard deviation	9.2	4.3	9.3	7.8
Maximum	6	4	19	13
Minimum	−34	−15	−24	−28
Range enclosing 90% of data	−29 to 0	−12 to 1	−18 to 14	−11 to 9

and spheres) it generally produced a mean of the right magnitude, but a large spread. It can be argued that at least part of the poor prediction accuracy was attributable to the way that the thermal data were chosen for this comparison but, even allowing for this, the agreement with finite differences was poor. This suggests that concept of using a mean thermal diffusivity in the precooling phase is possibly a cause of the problems. For milti-dimensional shapes the method gave predictions that both differed substantially from all the other methods, and were inaccurate compared to the experimental data. As previously stated this may be because the de Michelis and Calvelo method uses Plank's erroneous geometric factors for freezing whereas the other methods do not. Replacement by another concept such as EHTD or the mean conducting path is required.

The last stage in the proposed evaluation procedure was to provide details of thermal data and a worked example so that others can use the methods. This paper has presented all thermal data used. The papers of Pham (1984a) and Cleland and Earle (1982) contain worked examples.

DISCUSSION

THE ANALYSIS carried out has highlighted a number of issues which future research might address.

Firstly, when further data collection is planned the importance of including data with varying final product temperature should not be underrated because the prediction accuracy of a method may vary with this parameter. This is an area in which published data are scarce.

Secondly, numerical freezing calculations when compared with experimental data provide a substantial amount of information on experimental uncertainty. Problems in experimental data can often be detected by numerical calculations, so they should be considered an essential in all future studies. However, it is important not to assume that a finite difference calculation will always be accurate. Low accuracy thermal data used in a finite difference calculation, poor scheme formulation, and poor heat balances will lead to poor predictions, so cross-checking of finite difference results with experiments is still required.

Thirdly, simple comparisons of predictions with limited experimental data on their own do not allow the accuracy of a prediction method to be determined. Agreement with an accurate numerical method is just as revealing as agreement with experimental data. A prediction method must stand up against the full set of published data, not just part of it. Virtually all workers in the past, including the current authors, have been guilty of drawing rather sweeping conclusions on the basis of simple comparisons or limited data sets.

Fourthly, prediction formulae for shapes other than infinite slabs, infinite cylinders and spheres should be cau-

tious in use of Plank's geometric factors as these have been shown to be inaccurate (Cleland and Earle, 1979; 1982). One new form suggested has been termed an EHTD (equivalent heat transfer dimensionality), and is simply the relative rate of freezing of an object compared to that of a slab with the same characteristic dimension (Cleland and Earle, 1982). The EHTD concept allows separation of the freezing time prediction procedure into two components. (1) Slab: It is necessary to have a method to predict the freezing time for a slab accurately. There is probably still a need for research in this area. (2) Other shapes: The time for any other shape to freeze can be calculated from the slab time by a simple division by EHTD which is the relative rate of freezing of the actual shape compared to the slab. When faced by new shapes researchers need only look at the relativity of the freezing time of the new shape to that of a slab for different Biot numbers to establish EHTD values. This relativity can be established by running finite difference or finite element freezing calculations for the new shape, and for a slab frozen under the same conditions. The ratio of times is the EHTD. In this procedure any systematic error in thermal data cancels so experiments may not be needed. Finite elements allow irregular geometry to be studied in this fashion too. A very recent alternative to the EHTD has been proposed by Pham (1984b), which he terms a mean conducting path length. This can be determined in analogous fashion to an EHTD and may have advantages in some circumstances.

CONCLUSIONS

A SYSTEMATIC PROCEDURE for assessing freezing time prediction formulae is advocated.

An improved freezing time prediction formula is one which fits all available experimental data as well as can be expected taking into account data uncertainties, and which also makes predictions highly correlated with those from an accurate numerical method. It could also be simpler to use. Application of the procedure to four existing prediction methods established new insights of the strengths and weaknesses of each. Future research should address issues this study has highlighted – a need for experimental data in which the final product temperature is varied, cross-checking of all experimental data by numerical calculations, comparisons made over as wide ranges of experimental data as possible, and the development of simple shape factors for shapes other than slabs, cylinders and spheres.

SYMBOLS

- A – area (m^2)
- Bi – Biot number hD/k
- c – specific heat capacity ($J/kg°C$)
- C – volumetric specific heat capacity ($J/m^3 °C$)
- C_L – volumetric specific heat capacity of unfrozen material ($J/m^3 °C$)
- C_s – volumetric specific heat capacity of frozen material ($J/m^3 °C$)
- D – characteristic dimension (full thickness) (m)
- EHTD – equivalent heat transfer dimensionality
- h – surface heat transfer coefficient ($W/m^2 °C$)
- H – enthalpy (J/m^3)
- ΔH – enthalpy change in product between T_f and T_{fin} (J/m^3)
- i – time level in finite difference calculations
- k – thermal conductivity ($W/m°C$)
- k_L – thermal conductivity of unfrozen material ($W/m°C$)
- k_s – thermal conductivity of frozen material ($W/m°C$)
- L – latent heat of freezing (derived from ΔH by subtracting the sensible heat component) (J/m^3)
- M – mass (kg)
- n – space level in finite difference calculations
- r – correlation coefficient
- Δt – time step in finite difference calculations (s)
- T – temperature (°C)
- T_a – cooling medium temperature (°C)
- T_f – temperature at which freezing commences (°C)
- T_{fin} – final product center temperature at end of freezing process (°C)
- T_{in} – initial product temperature (°C)
- x – displacement (m)
- Δx – space step in finite difference calculations (m)
- ρ – density (kg/m^3)

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Effects of pH, Formulations and Additives on the Hydrogen Sulfide Content of Cooked Egg Mixtures

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ABSTRACT

The H_2S contents of cooked whole egg and albumen mixtures were highest from samples with pH around 7.5 and 7.0, respectively. The highest H_2S content was obtained for the cooked mixture containing a natural yolk to albumen ratio. The alteration of yolk to albumen ratio resulted in an alteration of pH for the mixtures and decreased the H_2S content of the cooked products. The addition of acetic acid, citric acid, Na_2EDTA, and polyphosphates at 0.1% or malic acid, monosodium phosphate, sorbic acid, succinic acid, and tartaric acid at 0.5% to the raw mixture reduced the H_2S content of the cooked samples.

INTRODUCTION

IN NEW PRODUCT DEVELOPMENTS or product improvements, flavor is one of the most important factors determining whether or not a particular product is acceptable to the consumer. As compared to other food items, the flavor chemistry studies of eggs have almost been neglected, although several sensory studies on eggs have been made and reported in the literature (Nath et al., 1973; Bemelmans and Noever de Brouw, 1974; Leutzinger et al., 1977).

Sulfur-containing volatiles contribute significantly to the overall flavor of foods. Tinkler and Soar (1920) showed that H_2S was produced when eggs were hard-boiled. The nature of this reaction has been investigated by Germs (1973), who reported that the production of H_2S from egg white was the result of a nonenzymatic reaction of the protein(s). Although H_2S contributes significantly to the flavor of eggs or egg products, it has also caused an off-odor and color problem in canned or heated proteinaceous foods, when this volatile compound was present at high concentration. For example, a greenish-black discoloration of the yolk in hard-cooked eggs was caused by the reaction of H_2S with the iron moiety of yolk (Tinkler and Soar, 1920; Romanoff and Romanoff, 1949; Germs, 1973). It is generally believed that heat-induced decomposition of the sulfur-containing proteins and amino acids of the food products yield H_2S. The formation of iron sulfide during the heating process was responsible for the darkening of heated proteinaceous foods.

Recently, Chen and Hsu (1981) reported that no difference in volatiles was found when egg mixtures were scrambled in a Teflon coated skillet, prepared by boil-in-bag method, or cooked in a bag in a microwave oven. Egg mixtures prepared by pan scrambling in a double boiler contained less H_2S than those mixtures prepared by the three previously described methods. They also reported that ingredients in scrambled egg mix had significant effects on flavor volatiles (Hsu and Chen, 1981). The omission of yolk increased H_2S and decreased carbonyls in the cooked mixtures and a reversed situation was observed in the mixtures which contained no egg white. This study was designed to investigate the effects of pH, formulations and additives on the production of H_2S in cooked egg mixtures. The effect of additives on H_2S was further studied with cysteine solution.

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MATERIALS & METHODS

SHELL EGGS were obtained from a single strain of White Leghorn hens from the Mississippi State University poultry farm, commercially washed and held in a 2–4°C refrigerator.

Experiment 1

Albumen was carefully hand separated from shell egg by an egg separator and mixed after pooling. Liquid whole egg mixture (mixture of 80.6% liquid whole egg, 12.2% water, 3.2% nonfat milk solid, and 4.0% corn oil) and albumen mixture (mixture of 82.1% albumen, 7.5% nonfat milk solid, 10.2% corn oil, and 0.2% NaCMC) were prepared separately as described by Chen and Hsu (1981). The pH of the prepared liquid whole egg and albumen mixtures was adjusted to approximately 5.0, 6.0, 7.0, 7.5, 8.0, and 9.0 with 6N NaOH or 6N HCl. Mixture samples (150g) were packed and heat-sealed in 15.24x15.24 cm cooking pouches (Sears Co., Chicago). The sample packages were then cooked in a Kenmore Model No. 747 microwave oven at "roast" setting for 3 min. Twenty grams of the cooked sample were blended with 40 mL distilled water in a Waring Blendor for 1 min prior to H_2S analysis (Luh et al., 1964).

The color of the cooked whole egg mixtures and the albumen mixtures was measured with a Model MC 10105 Macbeth colorimeter (Macbeth, Kolimorgan Co., Newburgh, NY).

Experiment 2

Mixtures of raw yolk and raw albumen at 0%, 16.67%, 33.33%, 50.00%, 66.67%, 83.33%, and 100% yolk were prepared. The pH of the mixtures was determined before microwave cooking as described in Experiment 1. Another set of mixtures was prepared by mixing cooked albumen with cooked yolk at the above mentioned yolk and albumen ratios.

Twenty grams of the mixture were blended with 40 mL distilled water and the pH of the blended samples was recorded before the H_2S analysis.

Experiment 3

Liquid whole egg mixture was prepared from fresh shell eggs as described in Experiment 1. Acetic acid, ascorbic acid, citric acid, disodium ethylenediamine tetracetic acid (Na_2EDTA), malic acid, polyphosphate (KENA), sodium phosphate (Primary), or sorbic acid were added separately at 0%, 0.1%, 0.5%, and 1.0% levels. The prepared liquid whole egg mixtures, with various levels of additives, were packed and cooked by microwave before H_2S analysis. The percent change in H_2S content in the treated samples was calculated against the control samples.

Experiment 4

Cysteine solution (3%) was prepared by dissolving L-cysteine hydrochloride monohydrate in distilled water and neutralizing it with 4N NaOH (Germs, 1973). Part of the prepared cysteine solution was heated in boiling water for 10 min. Selected additives, as described in Experiment 3, were added to 20 mL of the heated and nonheated cysteine solutions at 0.5% concentration. The additive-treated, nonheated cysteine solutions were then heated in the same manner. H_2S was determined as mentioned with the following modifications: liquid cysteine solution samples were not diluted with distilled water and the trapping time was reduced to 1 hr.

The effect of pH on H_2S production of the cysteine solutions was also determined by adjusting the pH of cysteine solutions to 2.5, 3.5, 4.5, 5.5, 6.5, 7.0, and 7.5 with 1N or 4N NaOH. Twenty mL each of the samples were heated in boiling water for 10 min and the H_2S content determined.

A complete random design of analysis of variance as described by Steel and Torrie (1980) was used to analyze the experimental

data. Duncan's New Multiple Range Test (1955) was used to separate the means.

RESULTS & DISCUSSION

Effects of pH and formulations on the H_2S of egg mixtures

The pH and formulations of egg mixtures affected the H_2S content of cooked samples (Tables 1 and 2). The pH for both the whole egg and albumen mixtures was 7.5 before adjustment. For whole egg mixtures, the H_2S content of the cooked sample was highest at a pH around 7.5; while the maximum H_2S production in the albumen mixtures occurred at pH 7.0. Adjusting the pH of whole egg mixtures to 5 and 6 significantly ($P < 0.05$) decreased the H_2S formation during cooking (Table 1).

The alteration of pH of the raw egg mixtures also affected the color attributes of the cooked products (Fig. 1). In general, products prepared from the mixtures with higher pH were darker and greener (higher Hunter "−a" values) in color than those of lower pH. Apparently, the greenish discoloration problem of the cooked egg mixtures was pH dependent and more serious for the albumen mixtures than for the whole egg mixtures. Results also suggest that this greenish discoloration can be controlled by the pH adjustment of the raw mixtures. According to Baker et al. (1967), the greenish-black discoloration of hard-cooked egg yolks was influenced by high cooking temperature, long cooking time, pH of yolk, long storage time of eggs before cooking and method of cooling the cooked egg.

Table 1—Mean H_2S content of cooked egg mixtures as affected by pH and formulation[a]

	H_2S Content (PPM)	
pH	Whole egg mixtures	Albumen mixtures
5.0	0.30Ac ± 0.00	0.48Be ± 0.01
6.0	1.47Abc ± 0.05	0.94Bde ± 0.02
7.0	3.88Aa ± 0.34	6.55Ba ± 0.08
7.5	4.68Aa ± 0.85	2.40Bbc ± 0.51
8.0	4.60Aa ± 0.74	3.05Bab ± 0.52
9.0	2.84Aab ± 0.70	1.64Bcd ± 0.15

[a] Mean of three determinations. Means within the same row followed by the same capital letters are not significantly different ($P > 0.05$). Means within the same column followed by the same lower case letters are not significantly different ($P > 0.05$).

Table 2—Hydrogen sulfide content of egg mixtures as affected by the yolk to albumen ratio

	Mean[a] H_2S content (ppm)	
Yolk:Albumen	Mixing raw yolk with raw albumen & cooking	Mixing cooked yolk with cooked albumen
0.00:100	3.53Ab ± 0.13	3.52Ac ± 0.12
16.67:833.33	3.81Ab ± 0.54	6.17Bab ± 1.10
33.33:66.67[b]	7.87Aa ± 0.39	7.71Aa ± 1.80
50.00:50.00	1.53Ac ± 0.36	5.59Bb ± 0.37
66.67:33.33	0.92Acd ± 0.35	3.41Bc ± 0.73
83.33:16.67	0.73Ad ± 0.04	1.70Acd ± 1.01
100.00:00.00	0.59Ad ± 0.10	0.59Ad ± 0.10

[a] Mean of three determinations. Means within the same row followed by the same capital letters are not significantly different ($P > 0.05$). Means within the same column followed by the same lower case letters are not significantly different ($P > 0.05$).
[b] Natural yolk to albumen ratio.

Effects of yolk to albumen ratio on the H_2S content of egg mixtures

The highest H_2S content was obtained for the cooked yolk and albumen mixture with the natural yolk to albumen (1:2) ratio. Increasing or decreasing the yolk to albumen ratio beyond the natural condition decreased ($P < 0.01$) the H_2S formation during the cooking process (Table 2).

Mixing cooked yolk with cooked albumen at various ratios did not yield a linear relationship in H_2S content. Again, the highest H_2S content in the mixtures was also obtained from the mixture with a natural yolk to albumen ratio (Table 2). Further studies of the yolk and albumen mixtures at various ratios showed that altering the natural ratio resulted in a change of pH in the mixtures (Table 3). The mixtures with natural yolk to albumen ratio (1:2) had a pH of 7.5. Increased amounts of yolk in the mixtures resulted in a decrease in pH. This alteration of pH might be partially responsible for the difference in H_2S content of the cooked mixtures.

Cooking the raw yolk and albumen mixtures together usually resulted in a higher pH ($P < 0.05$), when compared to the raw mixtures or the mixtures of cooked yolk and

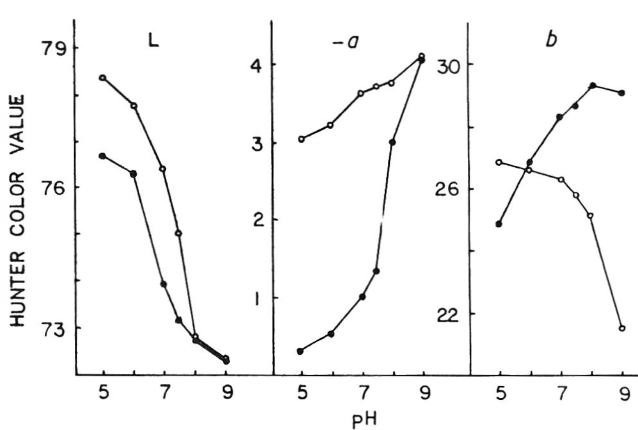

Fig. 1—Hunter color values of cooked egg mixtures as affected by pH: ○, whole egg mixture; ●, albumen mixture. Each point represents the mean of three determinations.

Table 3—Mean pH of blended egg mixtures[a,b]

	pH		
Yolk:Albumen	Raw yolk + Raw albumen	Mixing raw yolk with raw albumen & cooking	Mixing cooked yolk with cooked albumen
0.00:100	9.05Ba ± 0.07	9.82Aa ± 0.17	9.70Aa ± 0.00
16.67:83.33	8.33Cb ± 0.04	9.61Aa ± 0.13	9.05Bb ± 0.21
33.33:66.67[c]	7.75Cc ± 0.07	8.95Ab ± 0.21	8.33Bc ± 0.18
50.00:50.00	7.20Cd ± 0.14	8.38Ab ± 0.25	7.75Bd ± 0.07
66.67:33.33	6.80Ae ± 0.07	7.45Ac ± 0.21	7.25Be ± 0.21
83.33:16.67	6.50Bef ± 0.14	6.85Ad ± 0.00	6.83Ae ± 0.11
100.00:0.00	6.30Af ± 0.07	6.29Ad ± 0.02	6.29Af ± 0.02

[a] Mean of three determinations. Means within the same row followed by the same capital letters are not significantly different ($P > 0.05$). Means within the same column followed by the same lower case letters are not significantly different ($P > 0.05$).
[b] Twenty grams of egg mixtures were blended with 40 ml of distilled water.
[c] Natural yolk to albumen ratio.

Table 4—Hydrogen sulfide content of cooked liquid eggs as affected by selected additives at various levels

Selected additives	Mean[a] %[b] production		
	1.0%	0.5%	0.1%
Acetic acid	29.23Bbc ± 7.20	22.36Bb ± 1.57	76.10Ad ± 6.00
Ascorbic acid	265.67Aa ± 24.70	113.88Cc ± 27.02	193.67Ba ± 16.56
Citric acid	20.17Bbc ± 3.75	35.14Bab ± 5.54	85.14Acd ± 11.47
Malic acid	11.04Bc ± 1.80	24.66Bb ± 3.23	160.92Aab ± 31.51
Na_2EDTA	17.69Abc ± 7.41	16.07Ab ± 2.24	24.08Ae ± 4.66
Polyphosphates (KENA)	48.83Babc ± 4.93	42.94Bab ± 5.33	75.63Ad ± 9.78
Sodium phosphate (Primary)	73.19Aab ± 14.30	57.67Bab ± 4.04	127.35Abc ± 26.93
Sorbic acid	30.73Bbc ± 6.31	26.00Bb ± 2.00	161.72Aab ± 9.48
Succinic acid	44.10Bbc ± 6.51	62.87Bab ± 13.63	199.67Aa ± 4.73
Tartaric acid	16.97Bbc ± 8.49	15.50Bb ± 3.12	106.59Acd ± 18.93

[a] Mean of three determinations. Means within the same row followed by the same capital letters are not significantly different (P > 0.05). Means within the same column followed by the same lower case letter are not significantly different (P > 0.05).
[b] Percentages of H_2S production were obtained by comparing the treated samples with the non-treated controls.

Table 5—Hydrogen sulfide content of cysteine solution as affected by the selected additives at 0.5% level

Selected additives	Mean[a] %[b] reduction	
	Before cooking	After cooking
Acetic acid	59.31Aabc ± 13.29	24.34Bc ± 7.87
Ascorbic acid	75.61Aa ± 9.39	38.19abc ± 6.09
Citric acid	53.19Aabc ± 8.07	54.42Aab ± 5.84
Malic acid	46.90Aabcd ± 11.41	48.93Aabc ± 4.89
Na_2EDTA	36.84Abcd ± 11.37	31.72Abc ± 4.52
Polyphosphate (KENA)	22.66Ad ± 10.02	61.38Ba ± 5.53
Sodium phosphate (Primary)	32.13Acd ± 2.14	51.01Aabc ± 15.00
Sorbic acid	62.78Aab ± 8.93	59.49Aab ± 9.73
Succinic acid	36.94Abcd ± 3.72	48.53Aabc ± 16.25
Tartaric acid	60.34Aabc ± 9.52	44.44Aabc ± 7.64

[a] Mean of three determinations. Means within the same row followed by the same capital letters are not significantly different (P > 0.05). Means within the same column followed by the same letters are not significantly different (P > 0.05).
[b] Percentages of H_2S production were obtained by comparing the treated samples with the non-treated controls.

cooked albumen. The differences in pH became less definitive as the amount of yolk in the mixtures increased (Table 3).

Effects of selected additives on the H_2S production of cooked liquid whole egg mixtures of cysteine solution

The production of H_2S from liquid whole egg mixture during cooking can be altered by the addition of additives at various levels. The addition of acetic acid, citric acid, Na_2EDTA, and polyphosphates at 0.1% or malic acid, monosodium phosphate, sorbic acid, succinic acid, and tartaric acid at 0.5% to the raw mixture reduced the H_2S content of the cooked mixtures (Table 4). The addition of ascorbic acid to the mixture increased the H_2S content of the cooked mixtures at all three tested concentrations. Gravani (1969) reported that the addition of monosodium phosphate at 0.4%, EDTA at 0.07% or citric acid at 0.25% prevented the greenish-gray discoloration of cooked liquid eggs. Later, Gossett and Baker (1981) reported that acetic acid, 0.19%; citric acid, 0.17%; Na_2EDTA, 0.029%; malic acid, 0.22%; monosodium phosphate, 0.34%; and succinic acid, 0.27%, prevented the greenish-gray discoloration of cooked liquid whole eggs.

The effect of the selected additives on H_2S volatiles was further tested with cysteine solution (Table 5). In this part of the study, a trapping time of one hour and an incubation temperature of 24°C were used. The time and temperature selections were based on the results of our preliminary study (Fig. 2). Results from the studies involving the addition of selected additives, before and after the heating of the cysteine solution, showed that some of the selected compounds retarded the formation of H_2S, while others might act as H_2S chelating agents. The H_2S chelating effect

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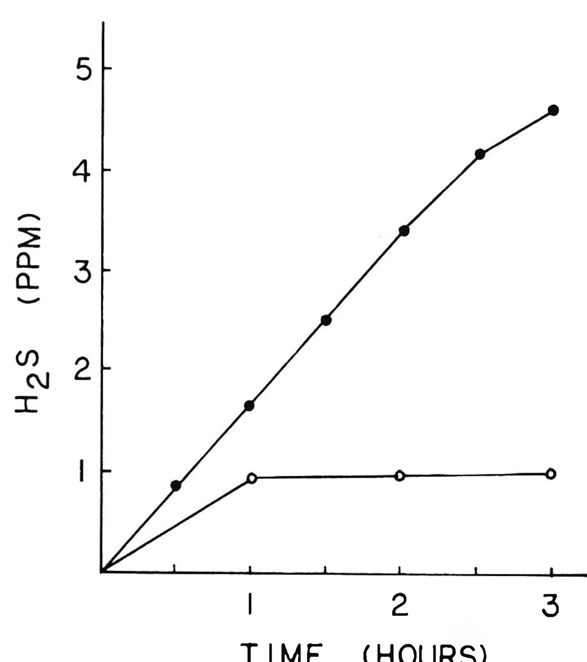

Fig. 2—Hydrogen sulfide assay of heated cysteine solution as affected by trapping time and temperature: •, 60°C; ○, 24°C.

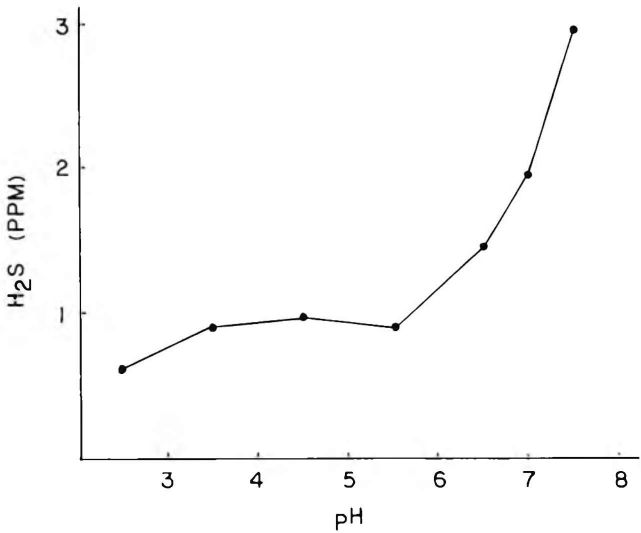

Fig. 3—Mean H_2S contents of heated cysteine solution as affected by pH values. Each point represents the mean of three determinations.

Simultaneous Curdling of Soy/Cow's Milk Blends with Rennet and Calcium or Magnesium Sulfate, Utilizing Soymilk Prepared from Soybeans or Full-Fat Soy Flour

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ABSTRACT

Mixed soy/cow's milk curds were prepared by simultaneous curdling of soy/cow's milk blends, utilizing rennet as cow's milk coagulant and calcium or magnesium sulfate as soymilk coagulant. The method produced curds of similar characteristics (compactness and yield), whether soymilk prepared from soybeans or full-fat soy flour was used. The effect of a number of process variables on curd characteristics was studied, utilizing a fractional factorial design. Generally, large changes in process variables (23 - 230%) produced relatively small charges in curd characteristics (1 - 16%). Protein contents of raw materials and proximate chemical analyses of pure and mixed milk curds, prepared under conditions yielding maximum curd compactness were determined. Protein recoveries in curd preparation were calculated.

INTRODUCTION

CHEESES of different types are much favored throughout the world, including developing countries. Although these products are known to possess high contents of good quality protein (FAO, 1970), they nevertheless have one important disadvantage as far as their use in developing countries is concerned: their high cost. Soybean products, on the other hand, represent an inexpensive and abundant source of protein, also of good quality (Wolf and Cowan, 1971).

For the above reasons, it appeared desirable to study extension of cheeses with soy proteins. Two possibilities were apparent for doing so: (1) curdle soy and cow's milk separately and mix the curds; and (2) mix soy and cow's milk and curdle the mixture simultaneously. Preliminary work on mixing separately prepared soy and cow's milk curds gave poor cheese because the curds had different structures, poor cohesion and broke on mixing. As a result of these tests, possibility (1) was excluded, leaving possibility (2) as the remaining option for preparing soy-extended cheeses of acceptable quality.

Previous reports on soy-extended cheeses include the work of Hang and Jackson (1967), who inoculated a 15/85 w/w mixture of skimmilk/soymilk with *S. thermophilus;* after incubation, the mixture was curdled by lactic acid produced by fermentation. Added rennet reduced the time from inoculation with starter to cutting the curd. Subsequently, Schroeder and Jackson (1971) applied a similar procedure to 25/75, 50/50 and 75/25 w/w mixtures of skimmilk/soymilk, again with addition of rennet. In all cases, cheeses of satisfactory quality were obtained, although it was found that the amount of skimmilk added had little effect on finished product texture and flavor, the latter being due to the strong flavor of soymilk. It might be added that soymilk obtained from soybeans by the traditional process (Smith and Circle, 1978) was used in both works, and that formation of soy and cow's milk curds was due mainly to isoelectric precipitation of casein and soy proteins by acid produced by fermentation, with added rennet only playing a minor role.

In this work, simultaneous curdling of soy/cow's milk blends of different proportions was studied, utilizing rennet for the cow's milk component and calcium or magnesium sulfate for the soymilk component.

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MATERIALS & METHODS

Preparation of soymilk from soybeans by the traditional process

Davis variety soybeans were soaked 24 hr in water at room temperature, using a water/bean ratio (w/w) of 1:4, the soak water was decanted and the beans washed. The soaked beans were mixed with water (1:9 w/w), ground 5 min in a Waring Blendor (high speed), and the resulting suspension filtered through cheesecloth. The residue was discarded; the filtrate, soymilk, was heated to 95°C and cooked at that temperature for 7 min. The final product was cooled and stored at 5°C until used. Protein content of the soymilk was determined using standard procedures (AOAC, 1970).

Preparation of soymilk from full-fat soy flour

Commercial full-fat soy flour was mixed with water (1:20, w/w) and the resulting suspension heated to 95°C and held at that temperature for 10 min, all with continuous stirring. The hot suspension was mixed vigorously (Waring Blendor jar, high speed) for 3 min. The resulting soymilk was cooled and stored at 5°C until used, and its protein content was determined by standard methods (AOAC, 1970).

Other materials

Raw cow's milk was obtained from a local dairy and stored at 5°C until used; its protein content was measured according to AOAC (1970) methods. Rennet was a commercial product in tablet form, of unspecified activity. Calcium and magnesium sulfates were chemically pure, laboratory-grade reagents, and did not contain water of crystallization.

Procedure for curd preparation

Each run was carried out as follows. Soy and cow's milks were mixed to obtain 2L of blend of the desired proportion (25/75, 50/50 and 75/25 soy/cow's milk, v/v) and blend acidity was adjusted to the required value using a 10% aqueous solution of lactic acid. The blend was heated to the required temperature and the curdling reagents, calcium or magnesium sulfate and rennet, were added in the required amounts, followed by thorough mixing to obtain uniform dispersion. The blend was allowed to repose at the required temperature (30 or 37°C) for the required length of time to allow curdling to occur. After the repose period, blend temperature was raised to 40 - 45°C to cook the curd. The curd was cut with a curd knife and transferred to a perforated wooden box containing 4 - 5 layers of cheesecloth. The whey was allowed to drain, then the warm curd, wrapped in cheesecloth, was placed in a hydraulic press and subjected to a pressure of 1000 psi for 24 hr. The curd was removed and evaluated.

Evaluation of curd characteristics

Two curd characteristics were measured: (1) consistency, determined utilizing a Koehler penetrometer with a universal ASTM

grease penetration cone; and (2) yield, calculated as weight of wet pressed curd obtained from 100L of milk blend.

Choice of independent variables

The following factors, which were believed to affect curd characteristics were chosen as independent variables and coded as follows: blend titratable acidity (A); amount of rennet added (B); type of soymilk coagulant employed (K); amount of soymilk coagulant added ($CaSO_4$ – C_a; $MgSO_4$ – C_b); curdling temperature (D); curdling time (E); and proportion of soy to cow's milk (P). Since a 2^n factorial experimental design was selected and since this requires use of each factor at two levels (low and high), corresponding levels chosen for the above factors were those considered to lie within current practice for manufacture of cheese from cow's milk and soybean curd from soymilk (Webb et al., 1974; Kosikowski, 1977; Davis, 1965; Smith and Circle, 1978; Schroeder and Jackson, 1971). It should be noted that in runs in which soymilk prepared from full-fat soy flour was utilized, one factor – proportion of soy to cow's milk – was studied at three levels.

Experimental design

A 2^n factorial design was selected. Since the complete design would have required performance of a larger number of experiments, which was considered impractical, it was decided to utilize a fractional factorial design instead. In this case, only part of the experiments required by the complete design are performed. A detailed description of the method is given by Fedener (1955).

Experimental design in runs utilizing soymilk prepared from soybeans

The experiment was carried out in 40 runs, with eight of the treatment combinations replicated to better ascertain experimental error. Table 1 lists factors used with corresponding levels. In the experimental design selected (Fedener, 1955), besides main effects, the following interactions were determined because they were believed to be important (refer to Table 1): A*B, A*C, B*K, and E*K. Higher order and the remaining two factor interactions were considered not significant. Due to the fractional design, other interactions were confounded with main effects (Fedener, 1955). In the design employed, C was nested within K. On each run, measurements were made of penetration and yield. The design matrix is given in Table 2, where the low level has been coded as –1 and the high level as +1. Replicate runs are shown in the table as "A" and "B". Results obtained were analyzed by Analysis of Variance techniques (Fedener, 1955).

Table 1—List of independent variables used in runs utilizing blends containing soymilk prepared from soybeans

Code	Factor	Low level	High level
A	Acidity	3.5% as lactic acid	5.0% as lactic acid
B	Rennet concentration	0.021 g/L of cow's milk in blend	0.042 g/L of cow's milk in blend
K	Type of soymilk coagulant[a]	Magnesium sulfate	Calcium sulfate
C	Amount of soymilk coagulant		
(a) $CaSO_4$		3.3 g/L of soymilk in blend	16.6 g/L of soymilk in blend
(b) $MgSO_4$		4.8 g/L of soymilk in blend	15.9 g/L of soymilk in blend
D	Curdling temp	30°C	37°C
E	Curdling time	1.5 hr	3.0 hr
P	Blend proportion of soymilk, v/v	25/75	50/50

[a] For this factor, $MgSO_4$ was coded as "low level" and $CaSO_4$ as "high level."

Experimental design in runs utilizing soymilk prepared from full-fat soy flour

The experiment was set up as a fractional factorial design with six factors. Of these, five were at each of two levels. The sixth factor consisted of three different soy/cow's milk proportions. The experiment was carried out in 48 runs, all replicated. Readings, at each factor combination, were made on penetration and yield. The same soymilk coagulant (magnesium sulfate) was used in all runs. The factors and their levels are given in Table 3, while the corresponding design matrix is listed in Table 4. Note that in the latter table, for the first five factors, low level has been coded as –1 and high level as +1; the sixth factor (P) was defined as follows: soymilk/cow's milk proportion 75/25, +1; 50/50, 0; 25/75, –1. Replicate runs are shown in Table 4 as "A" and "B". Besides main effects, the selected experimental design (Fedener, 1955) permitted calculations of the following interactions which were believed to be important: A*B, A*C, A*P, B*P, C*P, D*P, and E*P. Higher order and remaining second order interactions were assumed to be not significant. Due to the fractional nature of the design, other interactions were confounded with main effects. Results obtained were analyzed by Analysis of Variance techniques (Fedener, 1955).

Proximate analysis and protein balance calculations of pure and mixed milk curds

Proximate analyses of curds with minimum penetration, ob-

Table 2—Design matrix with corresponding results in runs utilizing blends containing soymilk prepared from soybeans

Run	A	B	C	D	E	P	K	Penetration[a]	Yield[b]
1	+1[c]	–1[c]	–1	+1	+1	+1	+1	140	12.00
2	+1	+1	–1	–1	–1	+1	+1	183	11.55
3	+1	–1	+1	–1	–1	+1	+1	103	10.65
4	+1	–1	–1	+1	+1	+1	–1	202	12.95
5	+1	–1	+1	–1	–1	+1	–1	174	12.30
6	+1	+1	+1	+1	+1	+1	–1	113	7.50
7	+1	–1	–1	+1	+1	–1	+1	170	11.55
8	+1	+1	–1	–1	–1	–1	+1	192	12.95
9	+1	–1	+1	–1	–1	–1	+1	197	22.40
10	+1	–1	–1	+1	+1	–	–1	203	12.10
11	+1	–1	+1	–1	–1	–1	–1	201	12.70
12	+1	+1	+1	+1	+1	–1	–1	204	9.50
13A[d]	+1	+1	+1	+1	+1	+1	+1	103	9.90
13B[d]	+1	+1	+1	+1	+1	+1	+1	168	10.65
14A	+1	+1	–1	–1	–1	+1	–1	194	11.30
14B	+1	+1	–1	–1	–1	+1	–1	183	9.50
15A	+1	+1	+1	+1	+1	–1	+1	142	11.75
15B	+1	+1	+1	+1	+1	–1	+1	150	12.00
16A	+1	+1	–1	–1	–1	–1	–1	195	12.75
16B	+1	+1	–1	–1	–1	–1	–1	203	12.75
17	–1	+1	+1	–1	–1	+1	+1	144	11.80
18	–1	+1	+1	–1	+1	+1	+1	213	14.70
19	–1	–1	+1	+1	–1	+1	–1	290	8.00
20	–1	+1	–1	+1	–1	+1	–1	158	12.35
21	–1	+1	+1	–1	+1	+1	–1	193	11.75
22	–1	–1	–1	–1	+1	+1	–1	194	11.50
23	–1	–1	–1	–1	+1	–1	–1	135	11;15
24	–1	+1	–1	+1	–1	–1	–1	173	11.90
25	–1	+1	+1	–1	+1	–1	+1	240	12.30
26	–1	+1	–1	+1	–1	–1	–1	138	9.85
27	–1	–1	+1	+1	–1	–1	–1	182	12.15
28	–1	+1	+1	–1	+1	+1	–1	180	12.20
29A	–1	–1	–1	–1	+1	+1	+1	156	12.95
29B	–1	–1	–1	–1	+1	+1	+1	196	12.95
30A	–1	–1	+1	+1	–1	+1	+1	194	14.05
30B	–1	–1	+1	+1	–1	+1	+1	108	10.65
31A	–1	–1	+1	+1	–1	–1	+1	145	12.00
31B	–1	–1	+1	+1	–1	–1	+1	165	13.80
32A	–1	–1	–1	–1	+1	–1	–1	124	10.20
32B	–1	–1	–1	–1	+1	–1	–1	135	11.50

[a] Penetration reported as penetrometer reading.
[b] Yield reported as percent wet pressed curd with respect to milk bland.
[c] High level coded as +1, low level coded a –1.
[d] Replicate runs coded as "A" and "B".

tained in both series of runs (i.e., utilizing soymilk from soybeans and from full-fat soy flour) were determined (AOAC, 1970). Considering these data, as well as protein contents of pure soy and cow's milks previously obtained (AOAC, 1970), protein balance calculations were carried out in order to determine protein recovery in curds.

RESULTS & DISCUSSION

Soymilk prepared from soybeans

Results of analysis of variance calculations are given in Tables 5 and 6 for penetration and yield, respectively. Due to unequal numbers of observations per cell, the design was not orthogonal, and hence different sums of squares do not add up to the total.

Factors found to be significant were K ($p < 0.05$), A*C ($p < 0.01$), A*P ($p < 0.05$) and B*K ($p < 0.05$) in the case of penetration, and D ($p < 0.05$), K ($p < 0.05$), A*B ($p < 0.10$), A*P ($p < 0.05$) and E*K ($p < 0.05$) in the case of yield. Most factors were significant at the $p < 0.05$ level; also, only two main effects – K, coagulant type and D, curdling temperature – were significant in these runs, both at the $p < 0.05$ level.

Effects of significant interactions on penetration and yield were calculated (Fedener, 1955) and found to be less than 1%.

Average penetration and yield values calculated, utilizing data for all runs, corresponding to low and high levels of significant main effects are shown in Table 7. Use of calcium sulfate as coagulant gave softer curds with higher average penetration and yield values (194 and 12.05%, respectively) than use of magnesium sulfate, for which corresponding values were 184 and 11.25%. On the other hand, an increase in curdling temperature from 30 to 37°C decreased average yield from 12.00 to 11.35%.

Nevertheless, Table 7 shows that overall percentage changes in both penetration and yield, caused by variation of type of coagulant and curdling temperature, were only of the order of 5 – 7%. Thus, variations in individual values reported in Table 2 were probably due more to experimental error than to significant main effects and interactions. It also means that it was possible to produce mixed soy/cow's milk curds by the method described, but that characteristics of these curds were not too sensitive to changes in experimental conditions, within limits of the experimental design employed. Given the small magnitude of these significant main effects, therefore, they will not be discussed further.

When studying main effects (an also interactions), it was found that in some cases, increasing penetration coincided with increasing yield, suggesting the possibility that the two parameters were correlated. To test this hypothesis, the correlation coefficient (r) was calculated (Snedecor and Cochran, 1967), utilizing data for all runs as reported in Table 2. The corresponding value calculated as 0.23, which indicated that the hypothesis was incorrect, and that penetration and yield were not correlated.

Table 3—List of independent variables used in runs utilizing blends containing soymilk prepared from full-fat soy flour

Code	Factor	Low level	High level
A	Acidity	3.5% as lactic acid	5.0% as lactic acid
B	Rennet Concentration	0.021 g/L of cow's milk in blend	0.042 g/L of cow's milk in blend
C	Amount of soymilk coagulant	4.8 gYL of soymilk in blend	15.9 g/L of soymilk in blend
D	Curdling temperature	30°C	37°C
E	Curdling time	1.5 hr	3.0 hr
P	Blend proportion of soymilk[a]	25/75 50/50	75/25

[a] Note that variable P, blend proportion of soymilk, was studied at three levels.

Soymilk prepared from full-fat soy flour

Analysis of variance calculations for the fractional factorial design employed in these runs are shown in Tables 8 and 9 for penetration and yield, respectively.

Factors found to be significant were A ($p < 0.01$), B ($p < 0.05$), O ($p < 0.01$), D ($p < 0.05$), E ($p < 0.05$), P ($p < 0.05$), A*C ($p < 0.10$), A*P ($p < 0.01$), B*P ($p < 0.05$), C*P ($p < 0.10$), D*P ($p < 0.01$) and E*P ($p < 0.01$)

Table 4—Design matrix with corresponding results in runs utilizing blends containing soymilk prepared from full-fat soy flour

Run	A	B	C	D	E	P	Penetration[a]	Yield[b]
1A[d]	+1[c]	−1[c]	−1	+1	+1	+1	188	12.70
1B[d]	+1	−1	−1	+1	+1	+1	122	12.45
2A	+1	+1	−1	−1	−1	−1	165	10.80
2B	+1	+!	−1	−1	−1	−1	112	12.20
3A	+!	−1	+1	−1	−1	−1	154	11.30
3B	+1	−1	+1	−1	−1	−1	124	11.15
4A	+1	+1	+1	+1	+1	+1	140	11.20
4B	+1	+1	+1	+1	+1	−1	112	11.80
5A	+1	−1	−1	+1	+1	0	95	11.35
5B	+1	−1	−1	+1	+1	0	163	10.55
6A	+1	+1	−1	−1	−1	0	120	11.25
6B	+1	+1	−1	−1	−1	0	150	10.35
7A	+1	−1	+1	−1	−1	0	211	11.50
7B	+1	−1	+1	−1	−1	0	193	10.75
8A	+1	+1	+1	+1	+1	0	98	11.00
8B	+1	+1	+1	+1	+1	0	118	11.50
9A	+1	−1	−1	+1	+1	−1	215	11.35
9B	+1	−1	−1	+1	+1	−1	193	11.05
10A	+1	+1	−1	−1	−1	−1	156	11.75
10B	+1	+1	−1	−1	−1	−1	133	11.65
11A	+1	−1	+1	−1	−1	−1	156	11.60
11B	+1	−1	+1	−1	−1	−1	210	12.00
12A	+1	+1	+1	+1	+1	−1	209	11.25
12B	+1	+1	+1	+1	+1	−1	217	11.60
13A	−1	−1	−1	−1	+1	+1	124	10.80
13B	−1	−1	−1	−1	+1	+1	103	11.20
14A	−1	+1	−1	+1	−1	+1	142	11.40
14B	−1	+1	−1	+1	−1	+1	102	11.75
15A	−1	−1	+1	+1	−1	+1	170	12.50
15B	−1	−1	+1	+1	−1	+1	126	12.15
16A	−1	+1	+1	−1	+1	+1	130	10.85
16B	−1	+1	+1	−1	+1	+1	109	10.90
17A	−1	−1	−1	−1	+1	0	161	12.70
17B	−1	−1	−1	−1	+1	0	212	12.95
18A	−1	+1	−1	+1	−1	0	82	11.80
18B	−1	+1	−1	+1	−1	0	93	11.05
19A	−1	−1	+1	+1	−1	0	169	12.00
19B	−1	−1	+1	+1	−1	0	195	12.10
20A	−1	+1	+1	−1	+1	0	194	12.10
20B	−1	+1	+1	−1	+1	0	175	12.65
21A	−1	−1	−1	−1	+1	0	107	12.65
21B	−1	−1	−1	−1	+1	−1	128	12.45
22A	−1	+1	−1	+1	−1	−1	78	11.85
22B	−1	+1	−1	+1	−1	−1	95	12.20
23A	−1	−1	+1	+1	−1	−1	97	12.50
23B	−1	−1	+1	+1	−1	−1	106	12.60
24A	−1	+1	+1	−1	+1	−1	184	12.45
24B	−1	+1	+1	−1	+1	−1	205	12.55

[a] Penetration reported as penetrometer reading.
[b] Yield reported as percent wet pressed curd with respect to milk blend.
[c] High level coded as +1, low level coded as −1.
[d] Replicate runs coded as "A" and "B".

for penetration; and A ($p < 0.01$), B ($p < 0.05$), P ($p < 0.01$), A*P ($p < 0.01$), D*P ($p < 0.01$) and E*P ($p < 0.05$) for yield. It is interesting to note that many more factors were found to be significant in these runs than in those utilizing soymilk from soybeans; also, most of these factors were significant at the $p < 0.01$ and $p < 0.05$ levels.

All main effects were found to be significant in the case of penetration, at least at the $p < 0.05$ level, while three of these – A, acidity, B, rennet dose and P, blend proportion of soymilk – were significant at the $p < 0.01$ level.

As in the previous runs, effects of significant interactions on penetration and yield were calculated (Fedener, 1955) and found to be less than 1% so that consequently, these will not be discussed further.

Average penetration and yield values calculated, utilizing data for all runs, corresponding to low and high levels of significant main effects are shown in Table 10. It is apparent that in all cases, large changes in independent variables (i.e., acidity, rennet dose, etc.; of the order of 23 - 230%) produced relatively small changes in both penetration and yield (1 - 16%). This same effect was observed in the previous runs utilizing soymilk prepared from soybeans. Also, average yield and penetration values obtained in the full-fat soy flour runs (Tables 4 and 10) were not much different from those obtained in the soybean runs (Tables 2 and 7).

These observations indicate that soymilk prepared from full-fat soy flour behaved similarly to that prepared from soybeans, at least as far as behavior in preparation of mixed curds by the present method is concerned. The proposed method, therefore, yielded curds of similar characteristics, regardless of soymilk origin.

Increase in acidity, magnesium sulfate concentration and curdling time increased penetration, resulting in softer curds, while increases in rennet concentration, curdling temperature and blend proportion of soymilk had the opposite effect, producing harder curds (Table 10). On the other hand, increases in acidity, rennet concentration and blend proportion of soymilk all resulted in a decrease in yield. Since they had little effect on both penetration and yield, as noted previosuly, however, these factors will not be discussed further.

The correlation coefficient between penetration and yield was calculated, using data obtained in all runs in which soymilk prepared from full-fat soy flour was utilized (Table 4; Snedecor and Cochran, 1967) and found to be 0.10. It was concluded that in these runs, as in those in which soymilk prepared from soybeans was used, penetration and yield were not correlated.

Proximate analysis and protein balance calculations of pure and mixed milk curds

Proximate analysis determinations of pure and mixed milk curds prepared under conditions giving minimum penetration, including protein contents of pure milks (experimentally determined) and milk blends (calculated) are given in Table 11. Soymilk prepared from soybeans had more than twice the protein content of that prepared from full-fat soy flour (5.3% and 2.0%, respectively). This difference may be explained as follows. In preparing soymilk from soybeans by the method described in this paper, raw soybeans containing native proteins were extracted with water; since native proteins possess high solubility, depending upon the water/bean ratio, high soymilk protein con-

Table 5—Results of analysis of variance calculations for penetration, in runs utilizing blends containing soymilk prepared from soybeans

Source of variation[a]	Degrees of freedom	Sum of squares	F Value[b]
A	1	164.1	0.17
B	1	91.6	0.09
C	2	1085.7	0.55
D	1	1283.1	1.29
E	1	364.6	0.37
P	1	68.6	0.07
K	1	4466.1	4.50**
A*B	1	528.5	0.53
A*C	2	12809.3	6.45***
A*P	1	7171.6	7.22**
B*K	1	5705.7	5.75**
E*K	1	54.8	0.06
Model	14	32254.8	2.32**
Error[c]	25	24817.7	
Total (corrected)	39		

[a] Please refer to Table 1 for coding.
[b] Significance level indicated as follows: *$p < 0.10$; **$p < 0.05$; ***$p < 0.01$.
[c] Error standard deviation = ± 31.5.

Table 6—Results of analysis of variance calculations for yield, in runs utilizing blends containing soymilk prepared from soybeans

Source of variation[a]	Degrees of freedom	Sum of squares	F Value[b]
A	1	392.2	0.64
B	1	334.0	0.55
C	2	1537.6	1.26
D	1	2635.4	4.31**
E	1	110.0	0.18
P	1	846.4	1.38
K	1	3760.0	6.15**
A*B	1	2405.4	3.93*
A*C	2	2931.0	2.40
A*P	1	2592.1	4.24**
B*K	1	874.3	1.43
E*K	1	1960.0	3.20**
Model	14	19018.1	2.22**
Error[c]	25	15293.5	
Total (corrected)	39		

[a] Please refer to Table 1 for coding.
[b] Significance level indicated as follows: *$p < 0.10$; **$p < 0.05$; ***$p < 0.01$.
[c] Error standard deviation = ± 1.24.

Table 7—Average yield and penetration values corresponding to low and high levels of significant main effects, in runs utilizing blends containing soymilk prepared from soybeans

	Average value at level				Percent variation in	
	−1[e]		+1[f]			
Parameter	Penetration[a]	Yield[b]	Penetration[a]	Yield[b]	Penetration	Yield
Coagulant type (K)[c]	184	11.25	194	12.05	+5.4%	+7.1%
Temperature (D)[d]	–	12.00	–	11.35	–	−5.4%

[a] Penetration reported as pentrometer reading.
[b] Yield reported as percent wet pressed curd with respect to milk blend.
[c] Percent variation in coagulant type not calcuable.
[d] Percent variation in temperature, +23.3%
[e] Low level coded as −1.
[f] High level coded as +1.

centrations are possible. Full-fat soy flour, on the other hand, has been considerably heat treated in order to inactivate enzymes and antinutritional factors, with a consequent sharp drop in protein solubility. Proteins present in full-fat soy flour soymilk therefore, were mainly those contained in insoluble flour particles suspended in water. Since a limit exists on the maximum amount of flour that may be suspended in order to yield a stable dispersion, a limit also existed on the maximum protein concentration of full-fat soy flour soymilk.

Milk curd protein content paralleled milk protein level (Table 11). Thus both soymilk curds ($CaSO_4$ and $MgSO_4$ precipitated) possessed higher protein contents than did the cow's milk curd. The cow's milk curd contained more fat (20.4%) than both soymilk curds (7.6% and 6.6% for the $MgSO_4$ and $CaSO_4$ precipitated curds, respectively), probably reflecting a similar difference in fat levels between soy and cow's milks (fat contents of these milks were not determined). Curd moisture contents were inversely proportional to total solids; due to its appreciably higher fat content, the cow's milk curd contained less moisture than either soymilk curd. The type of soymilk coagulant employed appeared to have little effect on curd proximate analysis.

Mixed milk curds prepared from soybean soymilk blends possessed higher protein contents than did those made from full-fat soy flour soymilk blends (Table 11). This is as might have been expected, in view of the higher protein content of the former with respect to the latter soymilk. Generally, curd protein content paralleled milk blend protein content. Thus, in the case of blends containing soymilk prepared from soybeans, protein content of both milk blends and curds increased with increasing soy proportion, while the opposite was true with blends containing soymilk prepared from full-fat soy flour. As in the case of pure milk curds, type of soymilk coagulant employed had little effect on mixed curd proximate analysis. As a whole, soybean soymilk curds exhibited lower fat contents than full-fat soy flour soymilk curds, probably reflecting parallel differences between raw material fat contents.

An interesting observation regarding mixed soybean soymilk curds is that all had lower protein contents than either 100% cow's or soymilk curds. This is important because, in view of what was noted in the previous paragraph, mixed curd protein levels should have been expected to lie between, and not below, those for corresponding pure milk curds. Since all mixed curds had lower fat contents than the pure cow's milk curd, this observation is probably best explained by the fact that protein recovery in mixed curds was lower than in pure ones (Table 12). This difference, in turn, indicates that curdling substances worked better when used with pure milks than when used

Table 8—Results of analysis of variance calculations for penetration, in runs utilizing blends containing soymilk prepared from full-fat soy flour

Source of variation[a]	Degrees of freedom	Sum of squares	F Value[b]
A	1	4508.6	8.04***
B	1	3383.5	6.03**
C	1	6594.1	11.75***
D	1	3162.3	5.64**
E	1	2697.0	4.81**
P	1	4735.9	4.22**
A*B	1	714.6	1.27
A*C	2	1930.4	3.44*
A*P	1	12315.0	10.97***
B*P	1	6076.5	5.42**
C*P	2	3029.1	2.70*
D*P	2	7679.3	6.84***
E*P	2	8964.5	7.99***
Error[c]	28	15710.2	
Total	47		

[a] Please refer to Table 3 for coding.
[b] Significance level indicated as follows: *p < 0.10; **p < 0.05; ***p < 0.01.
[c] Error standard deviation = ± 23.7.

Table 9—Results of analysis of variance calculations for yield, in runs utilizing blends containing soymilk prepared from full-fat soy flour

Source of variation[a]	Degrees of freedom	Sum of squares	F Value[b]
A	1	1728.0	30.96***
B	1	377.4	6.76**
C	1	28.8	0.52
D	1	12.6	0.23
E	1	33.0	0.59
P	2	665.0	5.96***
A*B	1	95.8	1.72
A*C	1	91.8	1.65
A*P	2	1676.7	15.02***
B*P	2	82.6	0.74
C*P	2	131.1	1.17
D*P	2	1579.9	14.15***
E*P	2	418.4	3.75**
Error[c]	28	1563.0	
Total		8484.2	

[a] Please refer to Table 3 for coding.
[b] Significance level indicated as follows: *p < 0.10; **p < 0.05; ***p < 0.01.
[c] Error standard deviation = ± 0.37.

Table 10—Average penetration and yield values corresponding to low, intermediate and high levels of significant main effects in runs utilizing blends containing soymilk prepared from full-fat soy flour

Parameter	Average value at level −1[h]		0[h]		+1[h]		Percent variation in	
	Penetration[a]	Yield[b]	Penetration[a]	Yield[b]	Penetration[a]	Yield[b]	Penetration	Yield
Acidity (A)[c]	137	12.00	–	–	156	11.90	+13.9%	−0.8%
Rennet dose (B)[d]	155	11.85	–	–	138	11.60	−11.0%	−2.1%
Magnesium sulfate dose (C)[a]	135	–	–	–	156	–	+15.6%	–
Temperature (D)[f]	156	–	–	–	135	–	−13.5%	–
Soymilk proportion (P)[g]	156	11.95	152	11.65	133	11.50	−14.7%	−3.8%

[a] Penetration reported as penetrometer reading.
[b] Yield reported as percent wet pressed curd with respect to milk blend.
[c] Percent variation in acidity, +42.9%.
[d] Percent variation in rennet does, +100.0%.
[e] Percent variation in amount of magnesium sulfate, +231.3%.
[f] Percent variation in temperature, +23.3%.
[g] Percent variation in soymilk proportion, overall, +200.0%.
[h] Low level coded as −1; intermediate level coded as 0 (only for factor P, soymilk proportion); high level coded as ±1.

in blends, probably because, according to the manner in which they were added (grams coagulant per liter of soy or cow's milk in blend), concentrations of these substances in blends were lower than in pure milks, in proportion to blend composition (e.g., in 50/50 blends, coagulant concentrations were 50% of those in pure milks). Presence of the opposite milk component probably also hindered action of a given milk coagulant, especially rennet.

Table 12 is interesting for a number of other reasons. It shows that protein recovery was the same, regardless of whether soymilk was curdled with calcium or magnesium sulfate, or cow's milk was curdled with rennet. With all mixed curds (soybean or full-fat soy flour soymilk blends), curd protein recovery decreased with increasing proportion of cow's milk; this probably indicated that cow's milk was more difficult to curdle than soymilk in blends. This may be so since curdling of cow's milk with rennet, being an enzymatic reaction, was probably more sensitive to curdling conditions than curdling of soymilk with calcium or magnesium sulfate, which is essentially a chemical reaction. For equal blend soymilk levels, higher protein recoveries were obtained with full-fat soy flour soymilk than with soybean soymilk. This observation is probably explained by the fact that full-fat soy flour soymilk, as previously noted, consisted of insoluble suspended particles, which were, therefore, quite easy to precipitate, while soybean soymilk had a much higher content of soluble protein which had to be precipitated by a chemical reaction.

Curds prepared using magnesium sulfate as soymilk coagulant exhibited somewhat higher protein recovery than those prepared using calcium sulfate. The former salt gave better and more thorough precipitation of proteins than did the latter. Interestingly, however, and as was previously noted, no effect of type of coagulant on protein recovery was found in the case of pure curds. Excluding experimental error, no explanation for this discrepancy is apparent at this time.

Table 11—Results of proximate analysis determinations for pure milks, milk blends and curds, in preparation of pure and mixed milk curds

System[a]	% Protein	% Fat	% Moisture
Pure milks and milk blends			
100% Soybean soymilk	5.3	–	–
100% Full-fat soy flour soymilk	2.0	–	–
100% Cow's milk	3.4	–	–
50/50 Soybean soymilk	4.4[b]	–	–
25/75 Soybean soymilk	3.9[b]	–	–
25/25 Soy flour soymilk	2.4[b]	–	–
50/50 Soy flour soymilk	2.7[b]	–	–
75/25 Soy flour soymilk	3.1[b]	–	–
Pure milk curds[c]			
100% Soybean soymilk, $MgSO_4$	34,9	7.6	54.3
100% Soybean soymilk, $CaSO_4$	35.3	6.6	53.8
100% Cow's milk, Rennet	29.9	20.4	45.6
Curds from blends containing soybean soymilk[c]			
50/50 Blend, $CaSO_4$	26.7	9.8	54.3
50/50 Blend, $MgSO_4$	26.6	9.4	54.7
25/75 Blend, $CaSO_4$	22.8	17.4	54.3
25/75 Blend, $MgSO_4$	20.4	17.4	55.9
Curds from blends containing full-fat flour soymilk[c]			
75/25 Blend, $MgSO_4$	16.9	23.0	57.0
50/50 Blend, $MgSO_4$	18.4	26.1	53.6
25/75 Blend, $MgSO_4$	19.6	28.4	50.2

[a] Key for blends: first number ferers to soymilk proportion.
[b] Calculated value, from blend composition and protein content of pure components.
[c] Type of coagulant used indicated after blend composition. In the case of blends, the other coagulant used was rennet, as noted in text.

SUMMARY & CONCLUSIONS

MIXED SOY/COW'S MILK curds can be prepared utilizing the method described in this paper. The method appears to work whether soymilk prepared from soybeans or full-fat soy flour is used, and curd characteristics (penetration and yield) are approximately the same in both cases.

In curds prepared from blends containing soybean soymilk, curd consistency increased (penetration decreased) by using magnesium sulfate as soymilk coagulant; in curds from blends containing full-fat soy flour soymilk, curd consistency increased with increasing rennet concentration, curdling temperature and blend soymilk proportion, and decreased with increasing acidity, magnesium sulfate dose and curdling time. In curds prepared from blends containing soybean soymilk, yield decreased when using magnesium sulfate as soymilk coagulant, and also decreased with increasing curdling temperature; in curds made from blends containing full-fat soy flour soymilk, yield decreased with increasing acidity, rennet concentration and blend soymilk proportion. In all of these cases, however, large changes in independent variables (23 - 230%) produced small changes in dependent ones (1 - 16%).

Due to their good consistency and compactness, best curds were judged by the researchers to be those with minimum penetration, because of ability of converting them to hard cheese. Conditions for obtaining these curds, from the above results, were as follows: titratable acidity, 3.5%; rennet concentration, 0.042 g/L of cow's milk in blend; magnesium sulfate concentration, 4.8 g/L of soymilk in blend; curdling tmperature, 37°C; curdling time, 1.5 hr; maximum proportion of soymilk in blend. Similarly, conditions for obtaining maximum curd yield were: acidity, 3.5%; rennet concentration, 0.021 g/L of cow's milk in blend; use of calcium sulfate as soymilk coagulant, independent of concentration; and minimum blend proportion of soymilk. Characteristics of best curds were: blends containing soymilk prepared from soybeans, penetration = 138 and yield = 11.51%; blends containing soymilk prepared from full-fat soy flour, penetration = 122 and yield =

Table 12—Results of protein balance calculations in preparation of pure and mixed milk curds

System[a]	Percent total protein recovered in curd
Pure milk curds[b]	
100% Soybean soymilk, $MgSO_4$	86.4
100% Soybean soymilk, $CaSO_4$	86.2
100% Cow's milk, rennet	87,1
Curds from blends containing soybean soymilk[b]	
50%50 Blend, $CaSO_4$	71.9
50%60 Blend, $MgSO_4$	73.6
25/75 Blend, $CaSO_4$	61.7
25/75 Blend, $MgSO_4$	69.0
Curds from blends containing full-fat soy flour soymilk[b]	
75/25 Blend, $MgSO_4$	83.1
50/50 Blend, $MgSO_4$	81.4
25/75 Blend, $MgSO_4$	76.9

[a] Key for blends: first number refers to soymilk proportion in blend.
[b] Type of coagulant employed indicated next to system composition. In the case of blends, the other coagulant used was rennet, as noted in text.

11.55%. Unfortunately, these characteristics could not be compared with those of pure (i.e., 100% soy or cow's) milk curds, since the latter were not prepared.

In runs utilizing soymilk prepared from soybeans, as in those in which soymilk manufactured from full-fat soy flour was used, curd penetration and yield were not correlated.

The order of decreasing protein content, in the case of pure milks, was as follows: soybean soymilk, cow's milk and full-fat soy flour soymilk. Curds prepared from these milks reflected the following characteristics: curd protein content paralleled milk protein content; curds prepared from soybean soymilk had higher protein, lower fat and somewhat higher moisture content than the cow's milk curd; and type of soymilk coagulant employed had little effect on curd proximate analysis.

On the other hand, the following was true concerning curds prepared from soy/cow's milk blends: curd protein content paralleled blend protein content; curds made from blends containing full-fat soy flour soymilk had lower protein, higher fat, and approximately the same moisture content as those prepared from soybean soymilk blends; all mixed curds had lower protein content than pure milk curds; and type of soymilk coagulant employed had no effect on curd protein content.

Protein recoveries in pure milk curds were approximately equal, whether cow's milk was curdled with rennet or soymilk was curdled with calcium or magnesium sulfates. Protein recovery in mixed curds was lower than in pure milk curds, decreasing with increasing blend proportion of cow's milk, and was higher in those prepared from full-fat soy flour soymilk. Type of soymilk coagulant had no effect on pure milk protein recovery; on the other hand, in mixed curds, higher protein recoveries were obtained when utilizing magnesium sulfate for this purpose.

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H_2S CONTENT OF COOKED EGG MIXTURES . . . From page 1045

was greater for polyphosphate at the 0.5% level. For acetic acid and ascorbic acid, H_2S formation prevention was more prominent than chelating. Ascorbic acid enhanced H_2S production in the liquid egg mixtures during cooking, while a H_2S reducing effect was observed for the cysteine solution.

Adjusting pH of the cysteine solutions between 2.5 and 5.5 did not affect their H_2S production during heating ($P > 0.05$). A further pH increase of the cysteine solutions to 7.5 significantly ($P < 0.05$) increased the H_2S content of heated solutions (Fig. 3).

At lower concentrations, H_2S probably contributes to the flavor of all heated proteinaceious foods; while at high levels, the objectionable odor of H_2S is detrimental to the flavor of such foods (Johnson and Vickery, 1964). Results reported in this study have shown that the H_2S content of egg mixtures can be altered through pH adjustment or the addition of additives at the proper levels.

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Quality Attributes and Retention of Selected B-Vitamins of Canned Faba Bean as Affected by Soaking Treatments

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ABSTRACT

Solutions of ethylenediamine tetraacetic acid and sodium bicarbonate were used to soak faba beans before canning. Soaking in ethylenediamine tetraacetic acid solutions only caused a slightly lighter bean color. Soaking in bicarbonate solutions increased both the drained weight and the softness of the cooked beans, and it also made the bean color darker. The profiles of loss of both thiamin and riboflavin during the canning process were determined. Riboflavin was lost primarily during retorting while thiamin was lost in several processes including heat pretreatment and soaking. Mechanism of loss for the two vitamins was mainly leaching; very little thermal destruction was observed. None of the soaking treatments affected the retention of these vitamins.

INTRODUCTION

LEGUMES are economical sources of protein, minerals, and B-vitamin complexes for a large population of the world. The faba bean (*Vicia faba* L.) is one such legume that has long been an important food to people of the Middle East. In the Orient, it is also popular, often being used in snacks, soups, and various other dishes.

Faba beans consumed in the home are often prepared through hydration and cooking to achieve the desired palatability. This method of preparation is very time and energy consuming. A canned product that simplifies or eliminates the preparation process in the home and offers long storage life may be of some value in the market place.

Experience from processing of other dry beans has shown that quality changes such as unfavorable color development (Swain, 1962; Furia, 1972; Luh et al., 1975) and water-soluble-vitamin losses (Lund, 1975) will take place during canning. These changes were further complicated by the additives, such as the disodium salt of ethylenediamine tetraacetic acid (Na_2EDTA) and sodium bicarbonate ($NaHCO_3$), used during processing (Daoud et al., 1977; Flora, 1980; Perry et al., 1976; Rockland et al., 1977; Silva et al., 1981). But effects of various processes and additives on the color, texture, and nutrient changes during the canning of faba beans, hitherto, have not been critically examined.

Thus, the purpose of this study was to investigate the effects not only of the processing steps, but also of some soaking treatments on the quality of canned dry faba beans.

MATERIALS & METHODS

Beans

Faba beans used in this study were purchased from Kuwait in 1978. They were sealed in a waterproof plastic bag and stored in a 4°C cold room to minimize quality change and insect damage. To obtain a sample of more homogeneous size, the beans were screened such that they would pass through a sieve with 3/8-inch openings (U.S.A. Standard Testing Sieve, Fisher Scientific Company) and remain as overs on a sieve with 1/4-inch openings. Beans with noticeable holes and cracked skins were discarded.

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Canning procedures

To eliminate the problem of germination during soaking, the beans were heat-pretreated in 80°C hot water for 3 min before soaking, then drained, cooled, and soaked in different solutions (Table 1) at a weight ratio of water to beans of 4 to 1 for 12 hr at 25°C. All soaked beans were drained, rinsed with tap water, and then blanched in a 95°C steam chamber for 3 min. Blanched beans (180g) were placed in a 303 x 406 enameled can, and brine solution containing 1.5% salt, 3% sucrose, and 0.1% monosodium glutamate was added up to 0.25 inches from the rim of the can (300 mL). The cans were covered with lids, exhausted in a steam chamber for 5 min, and sealed immediately by means of a semiautomatic sealer. The sealed cans were heat-processed at 115.5°C for 40 min. Faba beans processed under these conditions have been shown to be safe microbiologically (Abou-Dheir, 1980).

The canned beans were stored in a cool place (about 10 - 15°C) for 1 wk preceding quality evaluation and nutrient analysis.

Drained weight

The drained weight was determined by draining the canned beans for 2 min on an 8-in. standard #8 sieve and weighing to within 0.1g.

Quality evaluation

Color. Color was measured with a HunterLab color difference meter (CDM) model D25A-9 using a white porcelain plate with L = 92.34, a = –1.01, b = 0.91 as reference. Results were expressed in L, a, and b values.

Texture. Texture was measured with a Universal Instron Texture Machine (model 1122) using Ottawa extrusion cell having an area of 50 cm^2 and equipped with an eight-bar extrusion grid. The full-scale load was set at 100kg. Crosshead speed was 10 cm/min, and chart speed was 5 cm/min. Forty-five grams each of the canned beans were used, and measurements were made in triplicate. Results were expressed as the maximum force, in kilogram, required to extrude the beans.

Nutrient analysis

Thiamin. Thiamin contents were analyzed according to the AOAC thiochrome method (AOAC, 1980).

Riboflavin. Riboflavin in the beans was determined by using the AOAC fluorometric method (AOAC, 1980).

Statistical analysis

Analysis of variance was performed by using the SAS computer package to determine differences among treatments. When differences were observed, Duncan's multople range test was performed,

Table 1—Soaking solutions used in this study

Soaking solution	pH
Distilled water (control)	7.48
50 ppm Na_2EDTA	6.70
100 ppm Na_2EDTA	6.32
150 ppm Na_2EDTA	6.12
0.5% $NaHCO_3$	8.40
1.0% $NaHCO_3$	8.61
1.5% $NaHCO_3$	8.68

also by using the SAS package, to separate the effects of the treatments.

RESULTS & DISCUSSIONS

Drained weight

Drained weight of the canned products was affected by some of the soaking treatments (Table 2). Na_2EDTA-treated beans had the same drained weight as that of the control. However, beans treated with $NaHCO_3$ produced products with significantly greater drained weights than the control. Drained weight increased as the concentration of $NaHCO_3$ was increased with the exception of beans soaked in 1.5% $NaHCO_3$, the drained weight of which was not significantly different from that of the beans soaked in 1.0% $NaHCO_3$. Solubilization of pectic substances due to Na^+/Ca^{++} or Na^+/Mg^{++} exchange could contribute to the increase in drained weight. The fact that drained weight of beans soaked in 1.5% $NaHCO_3$ was not significantly different from that of beans soaked in 1% $NaHCO_3$ was believed to be caused by the greater loss of solids from the 1.5% $NaHCO_3$-treated beans during retorting. This reasoning was supported partially by the more viscous appearance of the final brine of this treatment as compared to those of the others.

Color

The effects of various concentrations of Na_2EDTA and $NaHCO_3$ on the color of canned faba beans are presented in Table 2. Incorporating 150 ppm Na_2EDTA in the soaking water produced canned beans with slightly lighter color than those soaked in plain water, as shown by the higher "L" value of the CDM measurements. The result agreed with those of Luh et al. (1975) and Flora (1980). The immobilization of the metal ions through binding to Na_2EDTA may be the cause for this change. Na_2EDTA concentrations less than 150 ppm did not cause a change in the "L" value. Neither "a" nor "b" values responded to the Na_2EDTA treatments.

The use of 1.0% and 1.5% $NaHCO_3$ in the soaking water darkened the color of canned beans, as indicated by their low "L" value. In addition, the "b" value was decreased by $NaHCO_3$ soaking treatments, indicating a reduction in yellowness. These results are similar to those of Kilgore and Sistrunk (1981) on blackeyed peas. Natural pigments, such as anthocyanins, were found to change color when blackeyed peas were soaked in alkaline solutions (Culver and Cain, 1952). These pigments could also contribute to the darkening of the $NaHCO_3$-treated beans in this investigation. No effect of $NaHCO_3$ on the "a" value was found.

Texture

Table 2 summarizes the effects of soaking rreatments on the texture of canned beans. The texture of $NaHCO_3$-treated beans was softer than those of the control and Na_2EDTA-treated beans. This is consistent with the findings of Varriano-Marston and Omana (1979). They suggested that the softening of the sodium salt-treated beans was due to the disruption of cell integrity caused by the ion-exchange reaction between the sodium ions and the divalent ions in the intracellular cement. In addition, the alkalinity of the $NaHCO_3$ solutions might also aid in hydrolyzing the cell wall, thus contributing to the softening of the beans.

It was also observed that, as the concentration of $NaHCO_3$ was increased, the texture of beans becomes softer. Since the ability to predict the texture of canned products at a given concentration of $NaHCO_3$ in the soaking water was of interest the data were subjected to regression analysis using two models, i.e., linear and quadratic. The independent variable, X, was the $NaHCO_3$ concentration, and the dependent variable, Y, was the maximum extrusion force measured.

A summary of the analysis of variance for the model is shown in Table 3. The quadratic model gave significantly higher R^2-values than the linear model. According to Table 4, the quadratic model could be expressed as:

$$Y = 55.27 - 51.54X + 17.74X^2$$

A good agreement was found between the extrusion forces as predicted by the quadratic model and those of the experimental data (Fig. 1).

None of the Na_2EDTA treatments showed any influence on the force required to extrude the canned beans when compared with control. Although the binding of intracellular divalent metal ions by chelating agent could also result in the softening of the beans (Varriano-Marston and Omana,

Table 2—Quality attributes of canned faba beans as influenced by various soaking solutions

Soaking solution	Drained wt (g)	HunterLab colorimeter readings L	a	b	Extrusion force (kg/45g)
Control	261.5^a	20.4^b	4.2^a	6.0^{bc}	55.3^c
Na_2 EDTA					
50 ppm	264.3^a	19.0^{ab}	5.5^a	5.7^{abc}	56.3^c
100 ppm	260.3^a	19.4^b	4.4^a	5.3^{abd}	56.7^c
150 ppm	262.2^a	22.2^c	4.8^a	6.4^c	57.6^c
$HaHCO_3$					
0.5%	273.2^b	18.5^{ab}	4.0^a	4.9^{ad}	33.7^b
1.0%	282.2^c	17.5^{ad}	4.7^a	4.5^{de}	21.7^a
1.5%	279.4^{bc}	15.9^d	4.4^a	3.6^e	17.8^a

$^{a-e}$ Means followed by the same letter (within each column) are not significantly different at the 5% level.

Table 3—Summary of analysis of variance for various regression models

Regression model	F Value	PR>F	R^2
Linear	151.45	0.0001	0.84
Quadratic	174.10	0.0001	0.93

Table 4—Test of significance for the parameters of quadratic regression equation

Parameter	Estimate	T for H_0: parameter = 0	PR>|T|	Std error of estimate
Quadratic model				
Intercept	55.27	37.04	0.0001	1.49
X	−51.54	−10.51	0.0001	4.90
X^2	17.74	5.62	0.0001	3.16

1979), the concentration of Na_2EDTA used in this study might have been too low to show this phenomenon.

Nutrient retention

Effect of canning operations. To ascertain if any changes in vitamin contents were taking place, the thiamin and riboflavin contents of faba beans were compared after each canning operation (Table 5). Both riboflavin and thiamin are water soluble, and some are expected to be lost during various steps of canning. It was observed that riboflavin content decreased significantly only at the retorting step. On the other hand, thiamin was vulnerable, not only to retorting, but also to other processing steps such as heat pretreatment and soaking. Strohecker and Henning (1965) reported that riboflavin occurred in natural products almost entirely in the combined forms, e.g., as riboflavin-5′-phosphoric acid ester (flavin mononucleotide), or linked to protein to become a constituent of "flavoprotein." Thus, it is possible that riboflavin in faba beans is so firmly bound to the protein matrix or other constituent that it is difficult to remove by leaching. Binding between thiamin and faba bean constituents must not be to the same extent as it is with riboflavin inasmuch as there was a 50% reduction in the thiamin content after soaking.

Because steam was the heating source in the blanching step, the contact between moisture and beans was minimized. This should reduce the leaching of water-soluble nutrients at this stage. Although the application of heat to the beans during blanching may also destroy thiamin, the effect was not observed in this study. Mulley et al. (1975) reported that binding of thiamin to the protein matrix and starch in naturally products could prevent the heat destruction of thiamin to a great extent. This may explain in part the heat-resistant properties of thiamin in our system. Furthermore, the short blanching time (3 min) used was not expected to cause a significant thiamin change.

Because of the drastic processing condition of a retort, a significant loss of riboflavin and thiamin was found. Most of the lost vitamins were recovered in the brine solution (Table 6 and 7) indicating that leaching was the major mechanism responsible for the vitamin decrease in the canned faba beans. The high temperature caused cell disruption and surface splitting of the beans which made them more susceptible to leaching losses. The mechanism that protected thiamin from heat destruction, as described in the blanching step, could also be effective during retorting.

Effect of soaking treatments. Thiamin retention of the canned beans was not significantly affected by either Na_2EDTA or $NaHCO_3$ treatments (Table 6). Although thiamin is known to be unstable under alkali conditions (Harris, 1975) the buffering capacity of the protein matrix and the acidic constituents of the beans may offset the alkalinity of the $NaHCO_3$ solutions for these treatments to show any effect.

Table 5—Test of significance for the vitamin changes during canning[a]

Canning operation	Thiamin[b] (μg/g)	Riboflavin[b] (μg/g)
Dry beans	4.40	4.40
Heat pretreatment	3.88*	4.26
Soaking	1.93*	3.99
Blanching	1.82	3.82
Retorting	1.49*	2.49*

[a] Values in this table are the means of thiamin or riboflavin contents for all treatments after the indicated process step.
[b] All values are on a dry weight basis.
* Nutrient contents are significantly different before and after the treatment at 5% level.

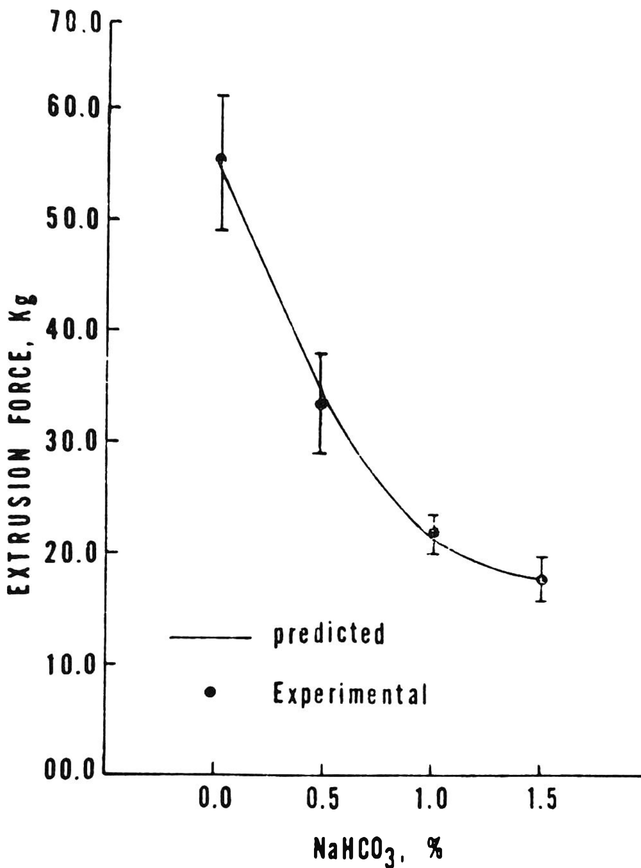

Fig. 1—Effect of the concentration of $NaHCO_3$ soaking solution on the texture of the canned faba bean. Bars denote standard deviation.

Table 6—Effects of soaking solutions on the thiamin content (μg/g) of faba bean at various stages of canning process[a]

Soaking solution	Soaked	Blanched	Canned	Brine
Control	2.53[b]	2.02[c]	1.60[d]	0.42
Na_2EDTA				
50 ppm	2.10[b]	1.84[c]	1.54[d]	0.41[e]
100 ppm	1.84[b]	1.70[c]	1.36[d]	0.45[e]
150 ppm	1.65[b]	1.80[c]	1.48[d]	0.41[e]
$NaHCO_3$				
0.5%	1.56[b]	1.71[c]	1.57[d]	0.35[e]
1.0%	1.76[b]	1.74[c]	1.52[d]	0.32[e]
1.5%	1.78[b]	1.96[c]	1.38[d]	0.25[e]

[a] Dry weight basis
[b-d] Means followed by the same letter are not significantly different at the 5% level.
[e] Single measurement

Table 7—Influence of soaking solutions on the riboflavin content (μg/g) of faba bean at various stages of canning process[a]

Soaking solution	Soaked	Blanched	Canned	Brine
Control	4.24[b]	3.80[e]	2.39[f]	1.51
Na_2EDTA				
50 ppm	3.68[cd]	3.66[e]	2.40[f]	1.74[g]
100 ppm	3.50[d]	3.74[e]	2.32[f]	1.70[g]
150 ppm	3.68[cd]	3.41[e]	2.45[f]	1.61[g]
$NaHCO_3$				
0.5%	4.07[bc]	4.21[e]	2.50[f]	1.72[g]
1.0%	4.29[b]	3.82[e]	2.75[f]	1.61[g]
1.5%	4.47[b]	4.12[e]	2.64[f]	1.53[g]

[a] Dry weight basis
[b-f] Means followed by the same letter are not significantly different at the 5% level.
[g] Single measurement

The retention of riboflavin also was not significantly influenced by the treatments during processing, except at the soaking stage (Table 7). Beans after soaking in Na_2EDTA solutions had slightly lower riboflavin contents than those soaked in other solutions. The cause for this decrease is not known. Even though precautions were taken to conduct the riboflavin analysis under subdued light, some light-induced degradation during analysis may still take place which may account for the detected differences.

CONCLUSION

SOAKING in Na_2EDTA solutions had very little effect on the color and texture of the canned faba bean other than a slight improvement of the bean color at the 150 ppm level. Soaking in $NaHCO_3$ solutions softened the canned beans drastically; however, it also darkened the product.

By tracing thiamin and riboflavin contents of the beans after each processing step, the profiles of change for these vitamins were outlined. Significant loss of thiamin occurred at the soaking, blanching, and thermal processing stages while major loss of riboflavin occurred only at the thermal processing stage of the canning process. The amount of thiamin and riboflavin recovered from the final brine indicated that these vitamin losses were due mainly to leaching instead of thermal destruction. Soaking in either Na_2EDTA or $NaHCO_3$ solutions did not result in additional loss in these vitamins. This result confirmed previous published works (Johnston et al., 1943; Rockland et al., 1977) that thiamin and riboflavin of some legumes, in their natural states, are not very susceptible to destruction by alkali and heat.

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Gossypol Removal and Functional Properties of Protein Produced by Extraction of Glanded Cottonseed with Different Solvents

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ABSTRACT

Cottonseed flakes were extracted with one of the following solvents: (A) hexane; (B) 1:1 mixture of 85% isopropanol and hexane; and (C) acetone followed by 1:1 mixture of isopropanol and hexane. Meal B had the lowest free gossypol content of 0.069%. The functional properties and the protein content of all the meals were similar. Ultracentrifugation did not reveal any change in the proportion of the protein fractions. Gel electrophoresis indicated minor differences.

INTRODUCTION

GOSSYPOL, a polyphenolic compound, is a constituent of cottonseeds (Murti and Achaya, 1975) and is toxic to monogastric animals (Berardi and Goldblatt, 1969). Several solvent-extraction methods have been used to reduce the gossypol content of cottonseed meal (Harris et al., 1947, 1949; Pons and Eaves, 1971; Damaty and Hudson, 1975; Canella and Sodini, 1977; Cherry and Gray, 1981). Although extraction with acetone or aqueous acetone produced cottonseed meal having low free gossypol content (Pons and Eaves, 1971; Damaty and Hudson, 1975) the flavor of the meal was objectionable (Alyevand et al., 1967). The use of acidic butanol for extraction left 0.07% free gossypol in cottonseed meal (Canella and Sodini, 1977), a level higher than the permitted level of 0.045% (Milner, 1980). Methylene chloride extraction of cottonseed meal reduced the free gossypol content to 0.013% with no adverse effect on the quality of the meal (Cherry and Gray, 1981).

Extraction of flaked cottonseed meal with isopropanol or aqueous isopropanol, and isopropanol-hexane mixture has been attempted both for oil extraction and for obtaining cottonseed meal of low gossypol content. Isopropanol (91%) was found to be an efficient solvent for extracting active (free gossypol) along with the oil (Harris et al., 1947). Rat and swine feeding tests of the isopropanol-extracted meal showed it to be superior to hydraulic pressed meal as a source of protein (Harris et al., 1947). Isopropanol has also been used in the preparation of fish protein concentrate (Toledo, 1974) and recently for the extraction of oil from soy flakes (Baker and Sullivan, 1983).

The object of this investigation was to determine how extraction of cottonseed meal with hexane, hexane-isopropanol mixture or acetone followed by hexane-isopropanol mixture affected the functional properties of the meal.

MATERIALS & METHODS

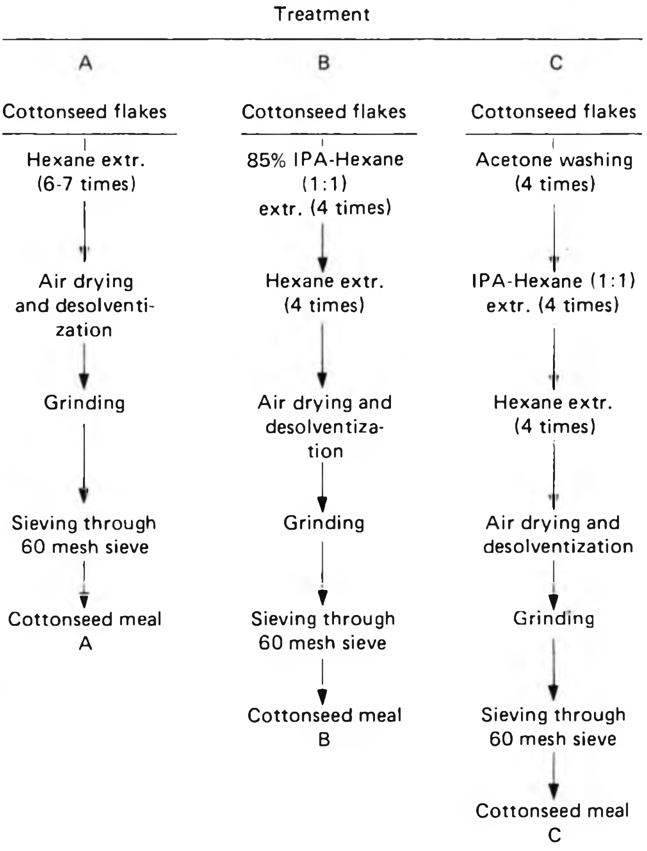
Materials

Cottonseeds of the variety *Varalakshmi* were obtained from the local market. The seeds were dehulled by flaking in a flaking roller (Model No. 6725, Akt.lebolaget, Kvarnmeskine, Malmo, Sweden), and then sieved to remove the husks and remaining fibers. The sieve-flaked samples were then extracted with one of the following solvents as indicated in Table 1: (A) hexane; (B) 1:1 mixture of 85% isopropanol (85 parts of isopropanol (IPA) and 15 parts of water) and hexane; and (C) washing with acetone followed by 1:1 mixture of isopropanol and hexane. For extraction, meal to solvent ratio of 1 to 10 was used in all cases. The meal samples were desolventized by drying at room temperature (∿ 28°C) and ground with an Apex Communiting Mill (Apex Construction Ltd., London) to pass through a 60-mesh size sieve. The fat content of the meals was below 0.5%. For brevity, the meal samples are designated as (A) hexane extracted meal; (B) meal extracted with 1:1 mixture of 85% isopropanol and water; (C) meal washed with acetone followed by 1:1 mixture of isopropanol and hexane.

Analytical methods

Moisture, total proteins, and total ash were determined by AOAC methods (AOAC, 1980). Total and free gossypol were determined by the methods of Pons et al. (1949; 1950). Bound gossypol was calculated as the difference between total and free contents. The available lysine content of the meals was determined by the procedure of Carpenter (1960) using 1-fluoro 2,4-dinitrobenzene (FDNB) reagent. The method of Dubois et al. (1956) was used to estimate total sugars in the ethanol extract using glucose as standard.

Table 1—Flow-sheet for preparation of cottonseed meal



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Meals were extracted with 70% ethanol for 2 hr on a water bath at 60°C. The in vitro digestibility index was determined by the method of Akeson and Stahmann (1964), using pepsin and pancreatin. The results are expressed as percent protein digested. Pepsin, pancreatin and 1-fluro 2,4-dinitrobenzene were from E. Merck, West Germany.

Disc polyacrylamide gel electrophoresis (PAGE) was carried out by the procedure described by Davis (1964) using 7.5% gel and 0.025M tris-glycine buffer of pH. 8.3. Cottonseed meal protein was extracted in 1M NaCl solution (meal to solvent ratio 1:10, extraction time 1 hr at room temperature), and the extract dialyzed against the buffer for 24 hr. About 100 μg of protein were loaded on the gel. Electrophoresis was carried out for 3 hr at a constant current of 3 mA/tube. The gels were stained with 0.1% Coomassie brilliant blue solution for 1 hr, then destained with a solution containing acetic acid, methanol and water (75:50:875).

The 1M NaCl-extract was dialyzed against 1M NaCl solution overnight and 1% protein solution was used in ultracentrifuge studies. The experiment was carried out with a Spinco Model E analytical ultracentrifuge, fitted with Rotor Temperature Indicator and Control (RTIC) unit and phase plate schlieren optics, at 60,000 rpm and at room temperature. Photographs were taken at different intervals of centrifugation. From the photographs, $S_{20,w}$ was calculated by the standard procedure (Schachman, 1959). Enlarged tracings were used to calculate the proportion of proteins in the extract.

The spectrum of the protein in dialyzed 1M NaCl-extract was recorded in the range 240 - 300 nm using Perkin-Elmer double beam recording spectrophotometer, Model 124.

Functional properties

Nitrogen solubility of each extracted meal in water and 5% NaCl solution was determined by the method described by Rahma and Narasinga Rao (1979). For water absorption capacity determination 15 mL distilled water was added to 1g of the meal in a weighed 20 mL glass centrifuge tube. The tube was agitated on a vortex mixer for 2 min, then centrifuged for 20 min at 4000g. The clear supernatant was decanted and discarded. The adhering drops of water were removed, and the tube weighed. Water adsorption capacity is expressed as the weight of water bound per 100g of dry meal. Oil absorption capacity was estimated in the same way as water absorption capacity except that 10 mL of refined groundnut oil were added to 1g of meal. The oil absorption capacity is expressed as mL of oil bound per 100g of dry meal.

Emulsifying capacity (EC) was determined by the method described by Webb et al. (1970). It is expressed as mL of oil emulsified per g of dry meal.

Table 2—Proximate composition of cottonseed meals obtained by extraction with different solvents[a]

Component	Meal A	Meal B	Meal C
Moisture (%)	7.2	7.6	7.3
Total protein (%)	47.9	49.7	51.6
Total suagrs (as glucose) %	16.0	14.2	15.2
Total ash (%)	7.6	8.2	8.9
Available lysine (g/16g N)	3.56	2.91	3.10
Total gossypol (%)	1.52	0.65	0.42
Free gossypol (%)	1.35	0.069	0.15
Bound gossypol (%)	0.17	0.58	0.27
Carbohydrates excluding Sugars (by difference)	14.70	16.09	16.70

[a] Average of three measurements

Table 3—Nitrogen solubility and in vitro digestibility of cottonseed meals obtained by extraction with different solvents[a]

Meal	Nitrogen solubility % H_2O	Nitrogen solubility % 5% NaCl	In vitro digestibility
A	25.9	66.5	80.2
B	18.5	54.1	75.4
C	25.4	75.9	81.8

[a] Average of three measurements

RESULTS & DISCUSSION

THE FREE GOSSYPOL CONTENT of the cottonseed meals prepared by extraction of the flakes with different solvents is given in Table 2. Hexane extracted meal contained 1.35% free gossypol compared with 0.069% when the extractant was a 1:1 mixture of 85% IPA and hexane. Pre-washing with acetone followed by extraction with 1:1 mixture of IPA and hexane gave a flour with 0.15% free gossypol. Gastrock et al. (1965) reported gossypol removal from cottonseed using hexane, acetone and water and obtained cottonseed flour with free gossypol content of 0.01 - 0.03%; their extraction procedures, however, were different from ours. Lawhon and Rao (1967) used acetone-cyclo-hexane-water mixture for extraction and by properly adjusting the water content of the mixture and the flakes obtained flours with 0.067% free gossypol and 50 - 57% protein. Cherry and Gray (1981) using methylene chloride with other suitable solvents obtained cottonseed flours with free gossypol content of 0.011 - 0.24% and protein content of 64 - 68%.

The proximate composition of cottonseed meals (Table 2) indicated there was not much variation in the protein, reducing sugar and total ash contents. IPA reduced slightly the available lysine content. Meals with reduced lysine had higher bound gossypol content. Polar solvents rupture the pigment gland and dissolve gossypol (Boatner, 1948). The probability that the protein will bind gossypol will be greater in such solvents. Thus dissolved gossypol in the aqueous IPA may be bound by the protein.

The solubility and in vitro digestibility of cottonseed flours are shown in Table 3. Solubility in 5% NaCl solution was higher than in water. Meal B had the lowest solubility. This may be due to denaturation of the proteins by aqueous IPA. Digestibility by pepsin-pancreatin system showed a trend similar to solubility. There was no difference in digestibility of meals A and C, and the values agreed with earlier reported values (Rahma and Narasinga Rao, 1983).

The water absorption capacity values (Table 4) show that nonaqueous IPA-hexane caused a slight increase and aqueous isopropanol-hexane a slight decrease in water absorption capacity. The use of both aqueous and nonaqueous IPA-hexane mixtures decreased the oil absorption capacity. In the case of 85% IPA-hexane solvent the decrease was marked (43%) but it was only 10% for the nonaqueous solvent treated sample. It is possible that the decrease in oil absorption capacity of 85% IPA-hexane extracted meal was due to the fact that this solvent was not as effective as the other solvents in removing the fat already bound to the meal. Also the 85% IPA-hexane extracted meal (B) may have been less lipophilic and less likely to absorb lipid than the other meals.

The EC of the samples was higher in 5% NaCl solution than in water (Table 4). Kinsella (1976) has reported that EC of a protein depends upon the solubilized protein. Since the nitrogen solubility of the meals in 5% NaCl solution was higher than in water, EC in 5% NaCl solution could also be expected to be higher. Perhaps for the same reason the EC of meal B was lower than that of the other two in water and 5% NaCl solution.

Table 4—Water and oil absorption and emulsification capacity of cottonseed meals obtained by extraction with different solvents[a]

Meal	Water absorption capacity g H_2O/100g meal	Oil absorption capacity mL oil/100g meal	EC mL oil/g meal H_2O	EC mL oil/g meal 5% NaCl
A	280	247	63	71
B	276	140	56	66
C	291	223	67	74

[a] Average of three measurements

The UV absorption spectrum of samples is given in Fig. 1. In 1M NaCl solution, meal A gave a spectrum with a maximum at 275 – 280 nm and minimum at 256 nm; meal B a maximum at 278 nm and minimum at 254 nm; and meal C a maximum around 278 nm and minimum around 250 nm. Whereas meals A and C gave a broad maximum, meal B gave a sharp maximum. The ratio of the absorbance at the maximum to that at the minimum had the following values: meal A, 1.09; meal B, 1.46; meal C, 1.17.

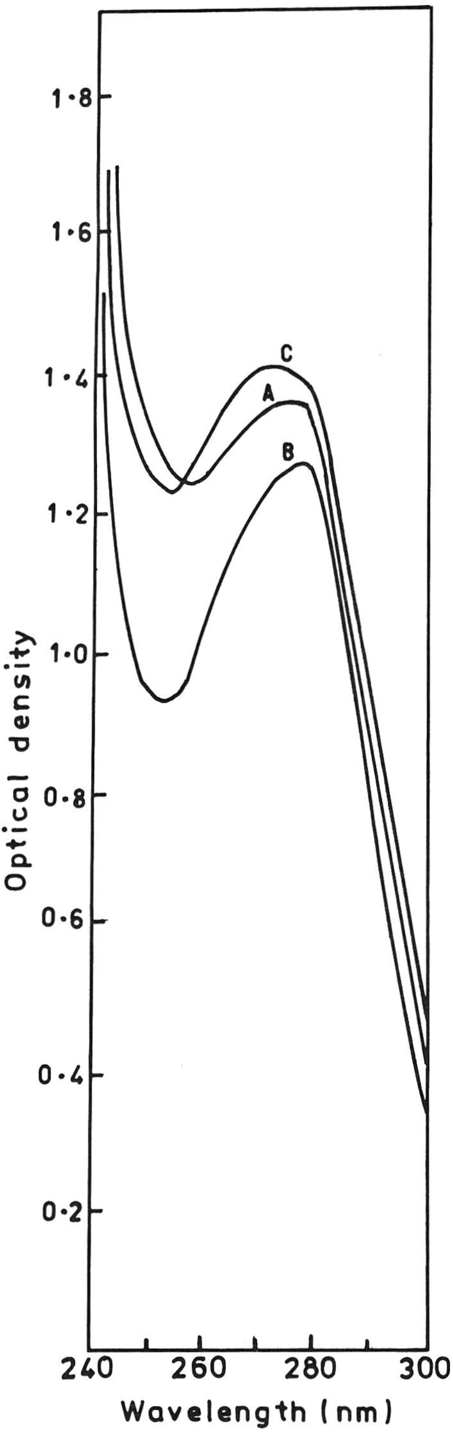

Fig. 1 – UV absorption spectrum of the protein samples (in 1M NaCl solution): (A) Flour obtained by hexane extraction; (B) Flour obtained by extraction with 1:1 mixture of 85% isopropanol and hexane; (C) Flour obtained by pre-washing with acetone and extraction with 1:1 mixture of isopropanol and hexane.

Proteins not conjugated with nucleic acid and other impurities give a ratio of 1.5 of absorbance at 280 nm to that at 260 nm (Layne, 1959). Meal B of present study had a value of 1.46, close to 1.5 suggesting that it was free from UV-absorbing impurities. This meal had also the lowest free gossypol content. Gossypol has absorption in aqueous solvents in the UV region with maxima at 292 – 295 nm and 385 nm (Maliwal et al., 1984).

The polyacrylamide gel electrophoretic pattern of the meals in 0.025M tris-glycine buffer of pH 8.3 is shown in Fig. 2. Meal A showed the presence of five major bands and two minor, fast moving bands. The major band has a relative mobility of 0.208 (Table 5). In meal B, the fast-moving bands were absent, and also the intensity of the two bands ahead of the major band was less, indicating partial dissociation. The relative mobility of the major band was low, only 0.16. This may be due to aggregation and formation of higher molecular weight fractions. The presence of an aggregate was also observed at the top of the gel (B). The pattern of meal C was almost similar to that of meal A. The major band had a relative mobility of 0.203 compared to 0.208 for the major fraction of the control.

Sedimentation velocity patterns of the 1M NaCl extract of cottonseed flour obtained by extraction with various solvents are shown in Fig. 3. The pattern in general consisted of 4 peaks, having $S_{20,w}$ values of 13.8S, 9.3S, 7.2S and 0.8S. The relative percent proportions of the four fractions were 3, 18, 46 and 33 respectively. The low molecular weight fraction gave a broad and diffuse peak characteristic of this class of proteins. The major peak in all cases was that of 7.2S protein. There was no change in sedimentation velocity patterns. PAGE experiments indicated minor variations in the protein composition. However, the chemical composition of the flour did not indicate any significant changes. There were some differences in the functional properties. Reddy et al. (1982) have reported that 7S fraction was the major protein in cottonseed.

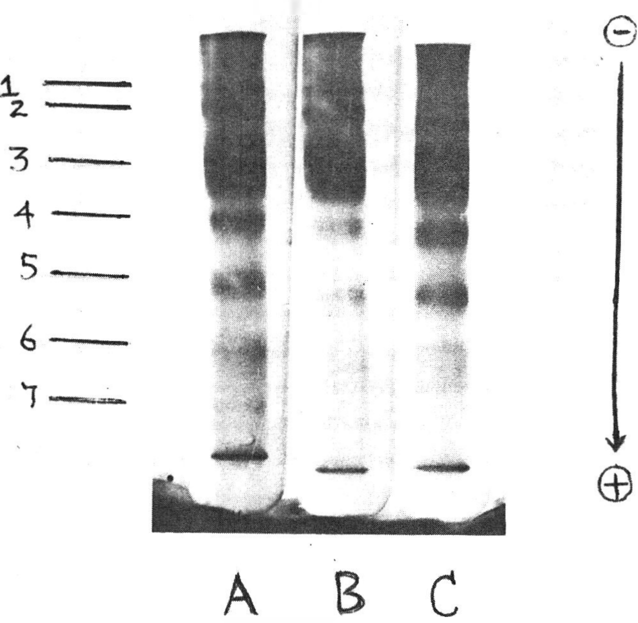

Fig. 2–Polyacrylamide gel electrophoretic pattern of the protein samples (in a 0.025M Tris-glycine buffer of pH 8.3): (A) Flour obtained by hexane extraction; (B) Flour obtained by extraction with 1:1 mixture of 85% isopropanol and hexane; (C) Flour obtained by prewashing with acetone and extraction with 1:1 mixture of isopropanol and hexane.

Table 5—Relative mobility of cottonseed meal proteins of different meals

No. of bands	Relative mobility		
	A	B	C
1	0.013	0.014	0.013
2	0.13	0.067	0.101
3	0.208	0.107	0.203
4	0.286	0.160	0.317
5	0.416	0.294	0.456
6	0.870	0.427	0.937
7	–	0.934	–

Extraction of cottonseed meal with a mixture of isopropanol and hexane removes 95% of gossypol left in the meal by extraction with hexane alone. Isopropanol-hexane extraction did not markedly alter the functional properties of the meal. The free gossypol content of 0.069% is slightly higher than the permitted level of 0.045%.

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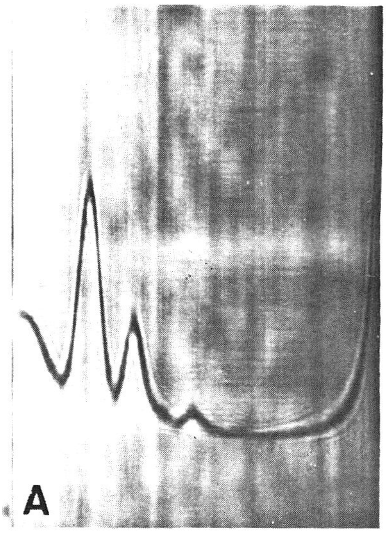

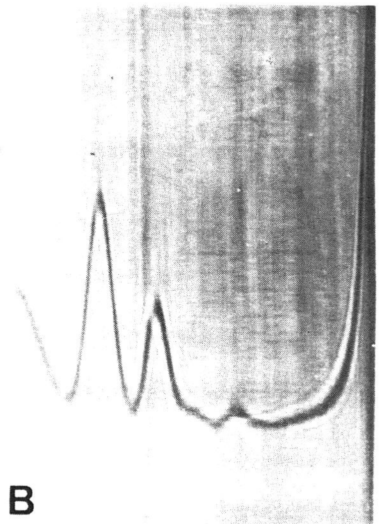

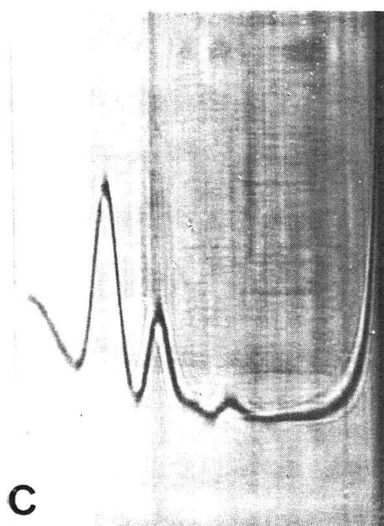

Fig. 3—Sedimentation velocity pattern of the protein meals (in 1M NaCl solution): (A) Flour obtained by hexane extraction. (B) Flour obtained by extraction with 1:1 mixture of 85% isopropanol and hexane. (C) Flour obtained by prewashing with acetone and extraction with 1:2 mixture of isopropanol and hexane. Sedimentation proceeds from left to right. The photographs were taken after 28, 29 and 31 min after attainment of 60,000 rpm for meals A, B, and C, respectively.

Thermocouple Grounding in Retort Pouches

WAYNE R. PETERSON and J. P. ADAMS

ABSTRACT

Four different types of thermocouples and three different grounding methods were evaluated to determine the most consistent and accurate temperature response in heat penetration tests of pouches in pressurized water and steam-air retorts. Criteria for evaluating the response were the residual sum of squares from nonlinear regression analysis and Fh and Fc values derived from the heat penetration data. Temperature measurement errors were found using both types of heating media for certain thermocouple types and grounding methods, while cooling data was found to be acceptable for most combinations. The best response was found with a thermocouple shielded with a stainless steel tube in which the measuring junction was electrically isolated from the sheath.

INTRODUCTION

ACCURATE MEASUREMENT of thermal processing parameters is necessary to obtain a valid calculated process time for the sterilization of canned and pouched foods. The validity of these parameters is directly related to the accuracy of the temperature measurements taken during a heat penetration test. Current thermocouple techniques used in the food industry have been developed for cans and glass containers and work well for those containers. With the advent of retort pouches, some temperature measurement problems have been observed with these standard techniques. Davis et al. (1972) experienced temperature measurement errors in retort pouches as large as 20°F when using copper-constantan wire thermocouples. They solved this problem by using enamel coated thermocouple wire and grounding the measuring junction of the thermocouple to the ground of the recording potentiometer. Pflug (1975) experienced similar problems and solved them in the same manner. Peterson and Adams (1983) used grounded thermocouples successfully; however, problems were experienced using ungrounded thermocouples (unpublished data).

Temperature measurement errors in retort pouches are usually found to be one of two types or a combination of both. The first and most common error appears as a tailing up or down of the straight line portion of the heating curve. This effect is similar to what is observed when a heating curve is plotted with an erroneous retort temperature. The other error encountered is erratic jumps up or down in the recorded temperature. These errors can be very severe, often resulting in recorded temperatures well above the actual retort temperature. The occurrence of these errors is inconsistant and the magnitude of the error will probably be different for each heat penetration test. If errors occur they usually become apparent as the temperature of the pouch contents approaches within 10-15°F of the retort temperature.

The purpose of this research was to examine the nature and occurrence of these temperature measurement errors in retort pouches, and to evaluate the effectiveness of thermocouple type and methods of grounding in alleviating these errors.

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MATERIALS & METHODS

TWO 10 x 9 x 1 inch polycarbonate slabs (Tuffac, Rohm and Haas Co., Philadelphia, PA) were used. Each slab was equipped with 4 holes (#50 drill, 0.07 inch diameter) at various heights in the slab (approximately +0.25, +0.10, 0.0, and −0.25 inches from center). The thermocouple holes were located in the infinite slab region of each slab as determined by comparing results from the exact mathematical solutions of the heat conduction equations for the infinite slab and parallelpiped geometries.

The polycarbonate slabs were processed in commercially available retort pouch material (0.5 mil PET/ADH/0.5 mil Al foil/4 mil PP) supplied courtesy of the American Can Company (Greenwich, CT). The pouch material was formed into a pouch with 1/4 inch wide impulse seals (14H/HTV, Vertrod Corp., Brooklyn, NY). The thermocouples were introduced into the pouch by means of stuffing boxes (C5.2, O.F. Ecklund, Cape Coral, FL). The final seal of each pouch was accomplished with a vacuum impulse sealer (Multivac M-3-II, Koch, Kansas City, MO). Each pouch was subjected to 29 inches of vacuum for at least 2 min prior to sealing. Following the vacuum seal, each pouch was given an additional cosmetic seal.

Thermocouple types

Four types of copper-constantan (Type T) thermocouples were used: (1) a 6-inch CNL needle thermocouple (O.F. Ecklund, Cape Coral, FL), consisted of 30 gauge thermocouple wire inside a 1/16 inch o.d. stainless steel tube. The measuring junction was formed by soldering the thermocouple wires and stainless steel tube together approximately 1/16 inch back from the end. (2) A 6 inch isolated CNL needle thermocouple (O.F. Ecklund, Cape Coral, FL) was identical to the standard CNL type except that the measuring junction was not in electrical contact with the stainless steel tube; therefore, the stainless steel tube was a true electrical shield. (3) 30- and (4) 40-gauge wire thermocouples (matched, Teflon coated copper and constantan thermocouple wire; Omega Engineering, Stamford, CT) were fused together to form the measuring junction. An extra copper wire of the same gauge was included in the fusion to provide a means of grounding the measuring junction. The 40-gauge thermocouple wire was very fragile; therefore, after approximately 6 inches of the wire was used, the 40-gauge wire was soldered to 24-gauge thermocouple wire (Thermo Electric, Saddle Brook, NJ). This soldered connection was secured to the polycarbonate slab using a cement made of polycarbonate shavings dissolved in dichloromethane. The wire thermocouples were held in place on the slab with a general purpose sealant silicon caulk (Dow Corning, Midland, MI). Previous experience had indicated that when more than one wire was extended through a stuffing box, a loss of vacuum in the pouch could result during processing. This problem was solved by using electrical shrink tubing in conjunction with silicon caulk at the point where the thermocouple wires entered the stuffing box. The wire thermocouples terminated outside the pouched polycarbonate slab at a female thermocouple connector (O.F. Ecklund, Cape Coral, FL), and the copper ground wire terminated at a small copper alligator clip.

Copper constantan Teflon coated thermocouple wire (24 gauge, Thermoelectric, Saddle Brook, NJ) was used from the measuring potentiometer into the retort vessel where it was terminated with a male thermocouple connector (C-6, O.F. Ecklund, Cape Coral, FL). The back of the male connectors and female connectors (wire thermocouples only) were filled with silicon caulk (Dow Corning, Midland, MI) to prevent water intrusion.

Grounding methods

Three different grounding methods were used: a retort grounding method, a potentiometric grounding method, and no ground. The retort grounding method was accomplished by attaching the ground wire from the 30- and 40-gauge wire thermocouples to the frame of the retort by means of an alligator clip. The nonisolated and isolated sheathed thermocouples were grounded to the frame of the retort by a copper wire with two alligator clips. One end of this ground wire was connected to the portion of the stainless steel sheath that extended outside the pouch, the other end was attached to the frame of the retort. Thus, for all the retort grounded thermocouples except for the isolated type, the measuring junction was grounded to the frame of the retort. The measuring junction of the isolated thermocouple was not grounded; however, the stainless steel shield surrounding it was. For the potentiometric grounding method, a copper wire was connected to the positive lead at the measuring potentiometer and grounded to the frame of the retort. For the potentiometric grounding method and no ground, the alligator clips on the 30 and 40 gauge wire thermocouples used for the retort grounding method were wrapped in glass cloth electrical tape.

Processing

The retort was designed and built at the University of Florida for processing institutional size retort pouches (Adams et al., 1983). One inch confining stainless steel racks constructed of 16 gauge stainless steel with 40% open area (3/8 inch holes in 9/16 inch staggered centers) were used. Two of the eight positions in the racking system contained pouched polycarbonate slabs, all other positions contained pouches filled with a 10% bentonite suspension. The pouched polycarbonate slabs were repeatedly processed until loss of vacuum occurred; at that point the slabs were repouched as described earlier.

Two heating media, flowing pressurized water and a steam-air mixture, were studied. For pressurized water processing the retort was loaded at ambient temperature and processing water, preheated to 270°F (132°C) was introduced into the pressurized retort. The slabs were processed at 250°F (121°C) with a come-up time of approximately 2-3 min. The processing water was recirculated at a rate of 110 gal/min (Reynolds number = 33,000), and an overriding air pressure of 10 psi was used (total system pressure of 25 psig). The polycarbonate slabs were processed until the slowest heating thermocouple had reached 245°F (118°C), the processing water was drained, and the retort was flooded with cooling water. The cooling water was circulated while being continuously vented and replenished, and required less than 10 min to reach a temperature within 1°F of the final temperature.

For steam-air processing a mixture of 90% steam and 10% air was used. The retort was filled with the pouched polycarbonate slabs at ambient temperature and processed in 100% steam for the first 1.5 minutes at which time air pressurization was initiated. After this venting and come-up, the slabs were processed at 250°F (121°C) with a total system pressure of 18.4 psig and at flow rate of 462 lb steam-air/hour (394 lb steam/hour) resulting in a Reynolds number of 4,800.

Experimental design

This experiment was designed to give comparisons between thermocouple type and grounding methods without confounding any main effects or interactions. Two assumptions were made for this design. The first assumption was that the two 1-inch slabs were identical as shown to be a valid assumption in another experiment (Peterson et al., 1983). Secondly, it was assumed that different positions in the same slab would result in the same Fh-value. Theoretically, this is a reasonable assumption.

The experiment was performed as a replicated block design to permit a statistical comparison of thermocouple types, grounding methods, and interactions of the two. The design yielded eight heating curves for each combination of thermocouple type and grounding method for both steam-air and pressurized water processing.

Data analysis

Temperature histories for both pressurized water and steam-air heating and for pressurized water cooling was recorded with a data logger (Digistrip II, Kaye Instruments, Bedford, MA). The potentiometer was equipped with 120dB common mode noise rejection circuitry. The data were analyzed using nonlinear regression analysis of the exponential function describing the straight line portion of the heating or cooling curve (Statistical Analysis System, release 79.6, SAS Institute, Cary, NC). The model used for heating data was:

$$(RT - T) = A*EXP(-2.302585*TIME/FH)$$

with TIME = Time of specific observation; RT = Retort temperature; T = Temperature observed at TIME; FH = Time required for straight line portion of heating curve to transverse one log cycle when plotted on semi-log graph paper; A = Intercept of straight line portion of heating curve at time zero. The model for cooling data was the same as that for heating data with the necessary adjustments.

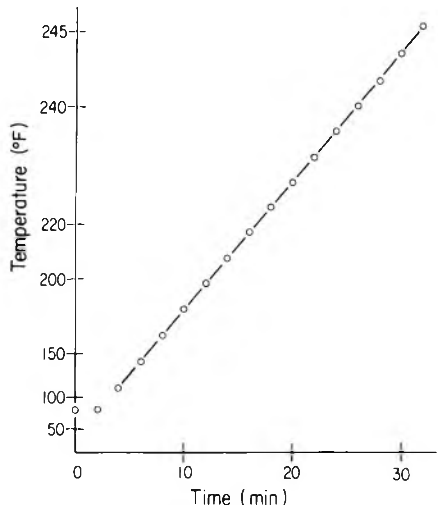
In all cases, 18 temperature values, each separated by 1 min, were analyzed for both heating and cooling. For heating, the last of the temperature values analyzed was 5°F below retort temperature, or approximately 245°F (118°C). The last of the cooling values analyzed was 10°F above the cooling retort temperature, or approximately 80°F (27°C). Heating and cooling retort temperatures were determined by averaging the temperature values of two thermocouples located at the exit of the retort racking system.

Statistical analysis of the resulting heat penetration data was accomplished using the Statistical Analysis System (release 79.6, SAS Institute, Cary, NC). Response variables analyzed were both Fh or Fc and the residual sum of squares generated by the nonlinear regression performed on each heating or cooling curve. The residual sum of squares was appropriate to use since all regressions contained an identical number of observations.

RESULTS & DISCUSSION

NONLINEAR REGRESSION ANALYSIS was utilized to equalize the influence of all the data points in determining the slope and residual sum of squares values. If a logarithmic transformation of the temperature differences and subsequent linear regression were performed, a low value (at high product temperatures) would have a greater influence in determining the slope value than a larger value of temperature difference. Since relative errors in accuracy of thermocouple values would be larger at low values of temperature differences, less accurate slope and residual sum of squares values would be obtained with linear regression.

Fig. 1 through Fig. 3 illustrate heating curves obtained for various residual sum of squares values. A very good straight line is obtained with residual sum of squares values of 0.57 or less (Fig. 1). Deviations from the straight line become apparent when residual sum of squares equaled 0.75 and 3.00 (Fig. 2 and 3, respectively). No figures with a residual sum of squares greater than 3.00 are presented because it was felt that Fig. 2 and 3 were unacceptable heating curves for polycarbonate slabs. The typical problem observed as the residual sum of squares increased was a tailing off or tailing up of the straight line portion of the heating curve as the measured temperature approached retort temperature (Fig. 2 and 3). Since values plotted were the differences between pouch and retort temperatures, plotting at a higher or lower retort temperature would straighten out these lines. The retort temperature measured by thermocouples in the retort was known to be accurate since those thermocouples were checked in an oil bath at 250°F (121°C) and found to agree within 0.3°F. This accuracy in measurements was coupled with the observation that the same thermocouple measuring a slab temperature in the retort did not consistantly give the same type or magnitude deviation from the straight line portion of the heating curve between replicate processing runs. Additionally, if an inaccurate retort temperature had been utilized in a given experimental run, all eight curves of that run would have been nonlinear. This was never the case since in each run there were at least two isolated and one nonisolated (either retort grounded or not grounded) thermocouples which did not show curvature. Additionally, since the rate of temperature change (slope) was the dependent parameter of interest, the effect of error in abso-



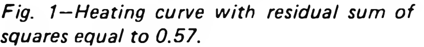


Fig. 1—Heating curve with residual sum of squares equal to 0.57.

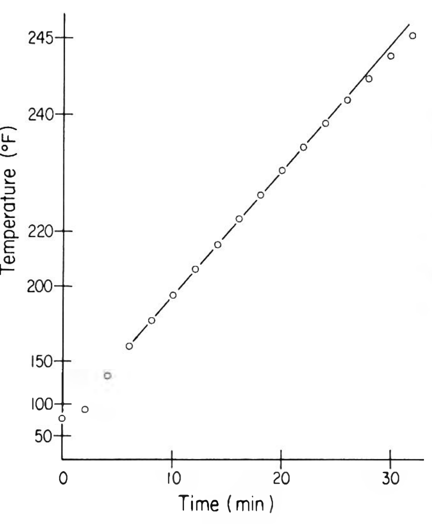


Fig. 2—Heating curve with residual sum of squares equal to 0.75.

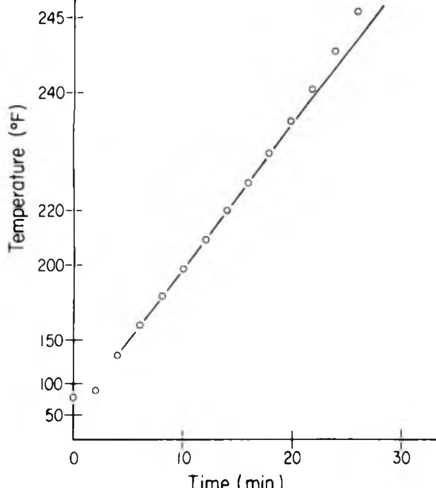


Fig. 3—Heating curve with residual sum of squares equal to 3.00.

lute temperature value measured (±1.5°F; potentiometer specifications) would be minimized. Thus, this effect was considered real and not just an artifact created by errors in temperature measurement.

Pressurized water heating

The residual sum of squares of the different thermocouple types and grounding methods for heating with pressurized water are presented in Fig. 4. Statistical analysis of the data revealed a significant interaction between thermocouple type and grounding method. The isolated thermocouple resulted in acceptable heating curves with low residual sum of squares in all cases, thus data obtained from this type of thermocouple was not dependent on the type or presence of ground. For the other three thermocouple types, grounding at the positive lead of the measuring potentiometer resulted in higher residual sum of squares with a high level of variation. This indicated that the heating curves obtained did not fit a straight line. The heating results using the potentiometric grounding method often showed temperatures near the end of processing as high as 15°F (8°C) above retort temperature. Obviously, grounding at the positive lead of the recording potentiometer led to more problems than it solved in the processing system used. This method of grounding was included in the experiment at the suggestion of the manufacturer of the recording potentiometer.

The nonisolated thermocouple type gave similar results with both the retort ground and no ground method since the residual of squares were within one order of magnitude of the isolated type of thermocouple. Grounding in the retort resulted in a smaller spread in the data than no ground; however, there was not a significant difference between the two methods. All but one of the nonisolated thermocouples without a ground gave an acceptable regression fit of the data. Past experience with nonisolated thermocouples which were not grounded had provided move variable results. Previous work with a 10% bentonite suspension and pouched seafood revealed the tailing data illustrated in Fig. 2 and 3 and also instances of instability in temperature measurement which were not observed in this experiment. A typical example of this instability found with a 1.0 inch thick pouched bentonite suspension is demonstrated in Fig. 5. The previous work indicated that the failures observed should have been observed in approxi-

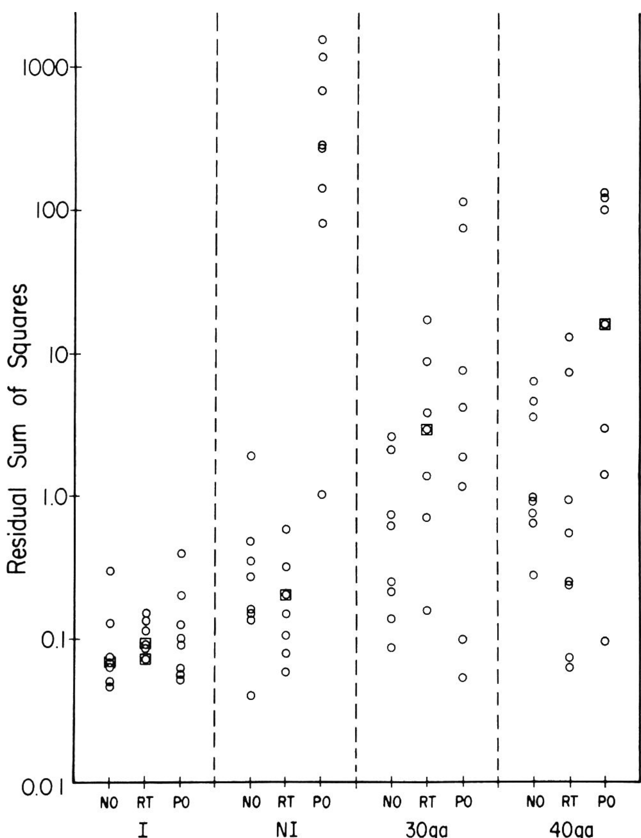


Fig. 4—Residual sum of squares obtained for pressurized water heating. I = Isolated thermocouple; NI = Nonisolated thermocouple; 30ga = 30-gauge wire thermocouple; 40ga = 40-gauge wire thermocouple; NO = No ground; RT = Retort ground method; and PO = potentiometric ground method. ◘ indicates 2 points at the same residual sum of squares value.

mately three out of eight heating curves; however, the retort was being utilized at a less frequent rate than in the current study. Hence, there was probably more dissolved and suspended rust in the heating water, possibly accounting for the more frequent occurrances of non-linear graphs. Subsequent work by Peterson and Adams (1983) with a 10% bentonite suspension revealed that the retort grounding method with the nonisolated thermocouple type essentially eliminated the problems.

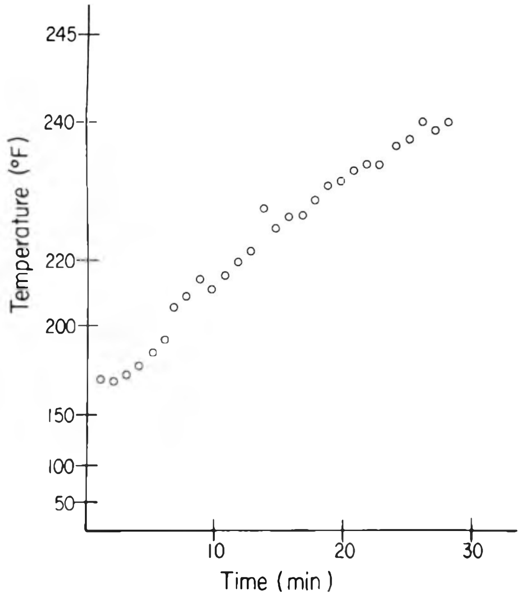


Fig. 5—Heating curve illustrating instability in temperature measurement.

The 30- and 40-gauge wire thermocouples gave similar results for each grounding method. In each case, the retort grounding method resulted in a greater degree of scatter in the residual sum of squares values than no grounding (Fig. 4). This was an unexpected result and was contrary to recommendations given by Pflug (1975). With wire thermocouples, the retort grounding method and the no ground method resulted in a large percentage of heating curves which did not have an acceptable fit of the regression equation, especially when compared to the isolated thermocouple type.

Statistical analysis using Duncan's multiple range test of the residual sum of squares for the pressurized water cook demonstrated no significant difference ($\alpha = 0.05$) between thermocouple types and grounding methods with one exception. The nonisolated CNL thermocouple coupled with the potentiometric grounding method was significantly different from all other combinations. Even though there was not a statistically significant difference, there was a practical difference between the methods as illustrated in Fig. 4. The low degree of scatter in the residual sum of squares for the isolated and grounded nonisolated thermocouples would suggest a greater confidence in these methods than those demonstrating the larger scatter in data.

In addition to how well the regression equation fit the heat penetration data, one must be concerned with the Fh-value obtained from the regression. An acceptable regression fit is inconsequential if the Fh-value obtained is erroneous. The mean Fh-values observed with the pressurized water cook (Table 1) should be approximately 20 min, as calculated from the thermal diffusivity of the polycarbonate slab (Peterson et al., 1983). The conclusions drawn from the Fh-values agreed quite well with those found in analysis of the residual sum of squares. The isolated thermocouple gave acceptable Fh-values independent of the grounding method used and the nonisolated thermocouple type gave acceptable results with no ground and the retort grounding method. The 40-gauge wire thermocouple with no ground gave an Fh-value close to that expected; however, the 95% confidence interval was large in comparison to the acceptable isolated and nonisolated thermocouple types. The 30- and 40-gauge wire thermocouples using the retort grounding method gave Fh-values lowever than what was expected. By far the best choice for thermocouple type is the isolated type, since it gave the expected Fh-value

Table 1—Mean Fh-values found for pressurized water heating

Thermocouple type	Grounding methods[a,b]		
	No ground	Retort method	Potentiometric method
Isolated	19.82 ± 0.35^{AB}	20.08 ± 0.31^{AB}	19.81 ± 0.43^{AB}
Nonisolated	19.12 ± 0.71^{AB}	19.87 ± 0.57^{AB}	9.56 ± 4.00^{D}
30 gauge	21.46 ± 3.05^{A}	18.05 ± 1.06^{B}	17.17 ± 3.93^{B}
40 gauge	19.50 ± 1.73^{AB}	18.08 ± 0.85^{B}	12.24 ± 2.04^{C}

[a] Mean Fh-value ± 95% confidence interval.
[b] Means with same letter superscripts are not significantly different (Duncan's Multiple Range Test, $\alpha = 0.05$).

with a very small 95% confidence interval independent of the type of grounding method used.

Steam-air cook

Fig. 6 contains the results of the residual sum of squares for each thermocouple type and grounding method for heating with 90% steam and 10% air as the processing media. These results are essentially identical to those found with the pressurized water cook. Overall, the steam-air cook residual sum of squares results demonstrated less scatter than the pressurized water cook. The isolated thermocouple type with the potentiometric ground method and the nonisolated thermocouple type with the retort ground method displayed more variation than with the pressurized water cook; however, the values were still, for the most part, acceptable.

The mean Fh-values for steam-air heating were less variable for isolated and nonisolated thermocouple types and generally more variable for the 30- and 40-gauge wire thermocouple types when compared to pressurized water heating (Tables 1 and 2). As in the pressurized water cook, the isolated thermocouple with any ground type and the nonisolated thermocouple with the retort ground method or no ground resulted in the expected Fh-value. The 30-gauge wire thermocouple with no ground gave an Fh-value close to the expected value of 20 min, but had a fairly large 95% confidence interval.

Cooling data analysis

The residual sum of squares for cooling data from the pressurized water cook, analyzed in the same manner as the heating data, are shown in Fig. 7. Unlike the heating results, cooling data did not show a significant interaction between thermocouple type and ground method. Additionally, there was not a statistically significant difference between main effects of either thermocouple type or ground method. All thermocouple types and grounding methods produced results of the same magnitude and variation (Fig. 7). The isolated thermocouple type did appear to have less variation in the residual sum of squares values compared to the other thermocouple types.

The residual sums of squares for cooling were generally higher than those found for heating with the isolated or nonisolated thermocouple, with the exception of the potentiometric grounding method with the nonisolated thermocouple. The length of time necessary to reduce the temperature of the retort to the final cooling retort temperature was the probable cause of the larger sum of squares. The retort dropped in temperature rapidly during the first portion of cooling but the rate of temperature reduction was greatly reduced as the retort approached the final cooling retort temperature. Because of this, the polycarbonate slabs were exposed to a changing retort temperature during cooling. A changing cooling retort temperature should result in a higher residual sum of squares than if the cooling retort temperature was reached in a matter of a few

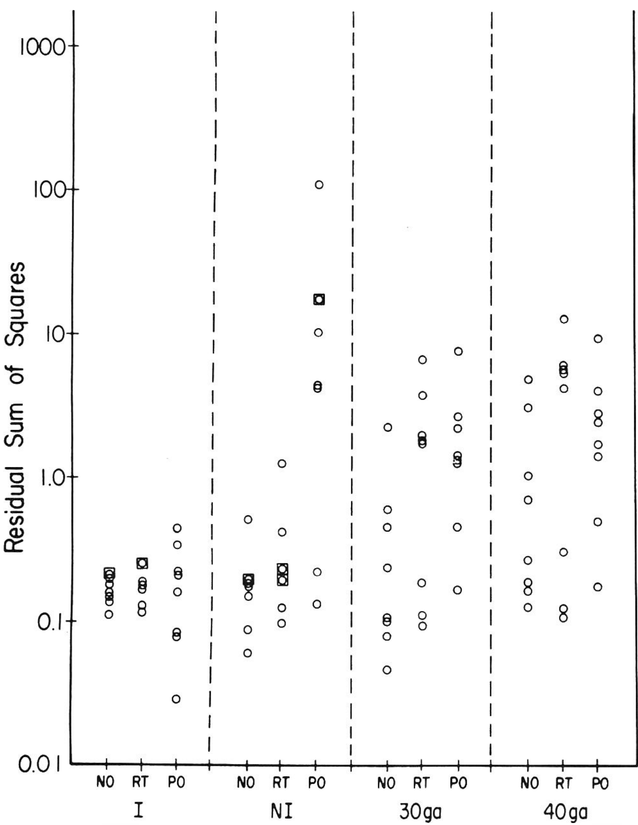


Fig. 6—Residual sum of squares obtained for heating with 90% steam, 10% air. I = Isolated thermocouple; NI = Nonisolated thermocouple; 30ga = 30-gauge wire thermocouple; 40ga = 40-gauge wire thermocouple; NO = No ground; RT = Retort ground method; and PO = potentiometric ground method. ◘ indicates 2 points at the same residual sum of squares value.

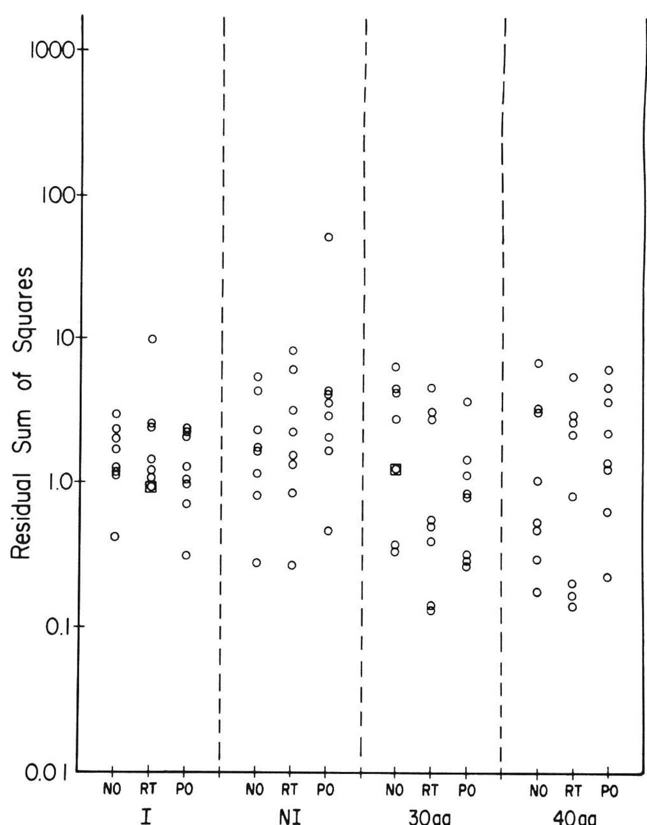


Fig. 7—Residual sum of squares obtained for cooling. I = Isolated thermocouple; NI = Nonisolated thermocouple; 30ga = 30-gauge wire thermocouple; 40ga = 40-gauge wire thermocouple; NO = No ground; RT = Retort ground method; and PO = potentiometric ground method. ◘ indicates 2 points at the same residual sum of squares value.

Table 2—Mean Fh-values found for steam-air heating

Thermocouple type	Grounding methods[a,b]		
	No ground	Retort method	Potentiometric method
Isolated	20.45 ± 0.24[CD]	20.52 ± 0.31[CD]	20.54 ± 0.26[CD]
Nonisolated	20.21 ± 0.59[CD]	20.38 ± 0.50[D]	16.54 ± 2.74[CD]
30 gauge	20.90 ± 1.04[BCD]	26.00 ± 4.80[ABC]	25.32 ± 5.18[AB]
40 gauge	21.78 ± 4.02[BCD]	29.46 ± 7.40[A]	28.83 ± 8.58[A]

[a] Mean Fh-value ± 95% confidence interval.
[b] Means with same letter superscripts are not significantly different (Duncan's Multiple Range Test, $\alpha = 0.05$).

minutes and remained constant throughout the majority of the cooling time.

Fig. 8 illustrates cooling data having a sum of squares value of 10.0 which resulted in a cooling curve providing an acceptable estimation of the Fc-value. Unlike heating where the last portion of the heating curve usually caused problems in Fh-value estimation, the cooling curves demonstrated a gradual bending consistent with a changing cooling retort temperature. Thus, all cooling curves were acceptable except for one obtained with the nonisolated thermocouple using the potentiometric grounding method.

The Fc values (Table 3) were all very close to the expected value with the exception of the nonisolated and 40 gauge wire thermocouples where the potentiometric grounding method was used.

SUMMARY & CONCLUSIONS

TEMPERATURE MEASUREMENT in retort pouches has been shown to display inaccuracies which arise during heating. These inaccuracies are usually most noticable in the later portions of heating, but were rarely observed during cooling operations. The errors encountered may be due to the electrical isolation of the pouch from the surrounding heating media or a combination of this electrical isolation and the static electricity characteristics of the pouch material (Pflug, 1975). The problem may also be related to the type of retort used and whether or not rust or hard water is present. The vessel of the retort used in this study was made of mild steel and rust was present. The presence of nonelectrically isolated thermocouple junctions in the retort did not influence the accuracy of two of the thermocouple types; so connectors could not be the cause of the inaccuracies observed. The potentiometer used did have common mode noise rejection circuitry, however, Klipec (1967) stated that the rejection circuitry did not totally eliminate the possibility of common mode noise. To minimize common mode noise, Klipec suggested shielding the thermocouple wire all the way back to the potentiometer and grounding the shield at the measuring junction of the thermocouple to insure that the shield was at the same potential as the measuring junction. None of the variables in the experiment utilized a shield from the measuring junction all the way back to the potentiometer. The problems observed were probably not common mode noise, since one would expect the same sort of problems with heat penetration tests in cans which is not the case. The problem must be directly associated with the nature or construction of the retort pouch and possibly some other factors, such as water hardness or suspended rust levels inherent to a particular processing system, since not all investigators have experienced these problems.

The 30- and 40-gauge wire thermocouples did not give adequate results with any of the grounding methods used.

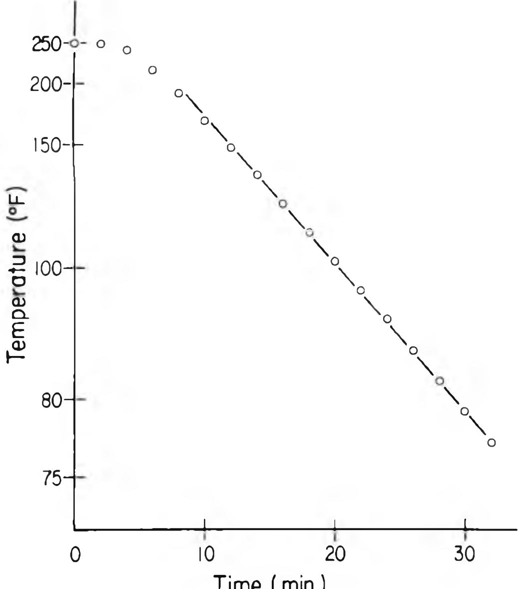


Fig. 8—Cooling curve with residual sum of squares equal to 10.0.

Pflug (1975) reported that problems of the nature described in this paper could be solved by running a ground wire from the measuring junction of a 30 gauge wire thermocouple to the case of the recording potentiometer. This is similar to the retort grounding method used in the present study since both the recording potentiometer and thermocouple were grounded to the frame of the retort. This study did not show this as an acceptable method for wire thermocouples with large retort pouches in the retort system used. The reason for this discrepancy is not known.

From the results presented it is apparent that the best thermocouple choice for retort pouch processing is one with a true electrical shield similar to the isolated thermocouple type. The results obtained from this type of thermocouple were independent of the grounding method and indicated that a cumbersome grounding wire in the retort is not necessary. The electrically shielded thermocouple provided equally good results with no ground or with both of the other two grounding methods used.

Table 3—Mean Fc-values found for water cooling

Thermocouple type	Grounding methods[a,b]		
	No ground	Retort method	Potentiometric method
Isolated	19.42 ± 0.21[B]	19.38 ± 0.22[B]	19.46 ± 0.23[B]
Nonisolated	19.61 ± 0.29[B]	19.93 ± 0.57[B]	21.39 ± 1.27[A]
30 gauge	19.23 ± 0.30[B]	19.46 ± 0.24[B]	19.86 ± 0.67[B]
40 gauge	19.74 ± 0.57[B]	19.99 ± 0.97[B]	21.30 ± 1.46[A]

[a] Mean Fc-value ± 95% confidence interval.
[b] Means with same letter superscripts are not significantly different (Duncan's Multiple Range Test, $\alpha = 0.05$).

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Influence of Sulfur Dioxide Generators on Red Raspberry Quality During Postharvest Storage

S. E. SPAYD, R. A. NORTON, and L. D. HAYRYNEN

ABSTRACT

Hand harvested 'Meeker' red raspberries were held at 4°, 14°, and 25°C for up to 12 days using two types of sulfur dioxide generating pads. Visual mold ratings were lower for fruits held with either SO_2 generator, but Howard Mold Count did not differ between the generators and the control fruits. Fruits stored with the generators were brighter and redder in color than controls when stored at 4°C. Irregular bleaching of anthocyanins from drupelets occurred when fruits were stored at 14°C or higher. Use of SO_2 generators is not recommended for fresh market fruit or nonheat treated processing fruit due to the bleaching and due to SO_2 residues in the fruit.

INTRODUCTION

BRAMBLE FRUITS have an extremely short shelf-life due to their fragile fruit structure and rapid rate of fruit respiration. The fruit are susceptible to attack by various fungi in the field and during handling and marketing. Holding raspberries at the lowest possible temperature above their freezing point is the best method for maintaining quality and reducing decay problems. Treatments such as CO_2 and SO_2 can be used as supplements to proper temperature management.

Storage of raspberries in 30% CO_2 for 4–6 hr resulted in raspberries of better quality than untreated raspberries after an unspecified period of commercial shipping (Winter et al., 1937). Raspberries developed off-flavors after 24 hr storage when held under constant 25% CO_2 atmospheres or an initial blanket of 45% CO_2 (Winter et al., 1939). An hour after removal from the high CO_2 the off-flavor disappeared. Using blackberries destined for the processing market, Morris et al. (1981) found that the use of 20 and 40% CO_2 partially offset the need for refrigeration to reduce postharvest quality losses. Ayres and Denisen (1958) tested several fungicides. They found that rimocidin at concentrations of 10 and 20 $\mu g/mL$ inhibited mold growth on raspberries.

Sulfur dioxide (SO_2) has been used for many years in the grape industry to inhibit the growth of fungi. In-package SO_2 generating systems were developed for release of SO_2 during storage and transit of table grapes (Gentry and Nelson, 1968; Nelson and Gentry, 1966). A fast-generation system was developed to replace the initial SO_2 fumigation prior to precooling, while a slow-generating system was developed to replace periodic treatments during storage. A dual release SO_2 generator was also developed which combined the fast and slow generating systems. The fast release generator consists of a paper sheet impregnated with sodium bisulfite which reacts with the moisture from the fruit to release SO_2. Maximum SO_2 concentrations generally occur during the first 24 hr. The dual release generator has sodium bisulfite encased in special plastic-lined paper pouches which gradually release SO_2 up to 12 wk.

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In Europe, sulfur dioxide is used to partially replace low temperature storage of small fruits prior to processing (Atkinson and Strachan, 1964). SO_2 is added to the fruits as sulfurous acid, calcium salts or as a gas and is removed by volatization during jam manufacture. It is not currently used in the United States for small fruits other than grapes. The effects of SO_2 generating pads have not been reported on raspberries.

The purpose of this study was to determine the influence of fast and dual release SO_2 generators on raspberry quality during postharvest storage at different temperatures.

MATERIALS & METHODS

1981 Studies

'Meeker' red raspberries were hand picked at the "fresh-market" ripe stage (bright red fruit color) from a 5-yr old planting at the Northwest Washington Research Unit, Mt. Vernon. Fruit was transported to the Irrigated Agriculture Research & Extension Center, Prosser, and held overnight at 4°C.

Bruised and moldy fruit were discarded as 150g of berries were sorted and placed into nonvented polyethylene containers (volume: bottom = 275 mL, top = 400 mL). Four storage treatments consisted of: (1) open container as an open control, (2) closed container as an untreated closed control, (3) closed container + 26 cm^2 of fast release SO_2 generator (FRG) (UVAS Quality Packaging, Inc., Antioch, CA 94509), and (4) closed container + 54 cm^2 of dual release SO_2 generator (DRG; 1 slow release cell + 54 cm^2 fast release). Raspberries were held for up to 12 days at 4°, 14°, and 25°C.

1982 Studies

Raspberries were harvested from the same planting that was used in 1981 and handled in a similar manner. Based on color at harvest, raspberries were sorted into bright red fresh-market and bluish-red processing maturities. Storage treatments in 1982 were the same as in 1981 except that only 4° and 14°C were used to compare the effect of SO_2 on the fresh market raspberries. Due to a limited amount of fruit, the processing ripe raspberries were stored at the intermediate temperature of 14°C. The 25°C storage was dropped in 1982 because of rapid raspberry quality deterioration at that temperature.

Laboratory analyses

The percentage of fruit with mold evident was visually rated by the senior author. For analysis raspberries were blended for 10 sec at high speed in a laboratory blender. Mold was determined by the Howard mold count (HMC) technique (NCA, 1980). Puree color was determined with a Hunter Color and Color Difference Meter standardized to a dark red plaque ("L" = 23.3, "a" = 20.4, "b" = 7.0). For total anthocyanin (TAcy) concentration, 25g puree were blended with 100 mL EtOH:HCl (pH 1.0) for 2 min at high speed and filtered through Schleicher and Schuell No. 588 fluted filter paper. Absorbance at 520 nm was determined with a Beckman DU Spectrophotometer equipped with a Gilford Modernization System. TAcy concentration was calculated as absorbance at 520 nm × dilution factor ÷ weight of sample.

Percent soluble solids of the puree was determined at 20°C on a Bausch & Lomb Abbe refractometer. Acidity, expressed as % citric, was measured by titrating a 3g sample of puree diluted to 100 mL with distilled water to pH 8.4 with 0.1N NaOH. The pH was determined using a Beckman pH meter (Model 3500) which was standardized to pH 4 and 7.

In 1981, sulfur dioxide was extracted from the fresh market

fruit held at 4 and 14°C by distilling 20g of puree in a Cash steam distillation apparatus. The distillate was collected in 0.02N I_2 and SO_2 concentration determined by back titration with thiosulfate as described by Amerine and Ough (1980).

Experimental design

The experimental design in 1981 and 1982 was a randomized complete block with three replications. Data were subjected to analysis of variance and F-test. Mean separation was by Duncan's multiple range test, 5%.

RESULTS

1981 studies

Raspberries stored with the dual release SO_2 generator (DRG) had the lowest ratings for visual control of mold and percent mold as indicated by Howard mold count (HMC) (Table 1). As expected, mold increased with increased storage time and temperature. Desiccation of the open control raspberries and softening of the SO_2 treated fruits were observed when visual mold was rated.

Mold concentrations were reduced for up to 12 days when raspberries were stored with the DRG (Table 2). The fast release SO_2 generator (FRG) reduced mold growth for up to 3 d which corresponds to the expected period of highest SO_2 concentrations. However, based on HMC levels neither the DRG or FRG treatments held mold growth at an acceptable level. Only the initial fruit had acceptable levels (not more than 60% positive fields) of mold filaments by HMC.

Use of DRG and FRG reduced the rate of loss of soluble solids for up to 12 d and 6 d, respectively (Table 2). Soluble solids reductions in raspberries from open controls were masked by desiccation.

In general raspberry pH increased with increased storage time and temperature (Table 1). Raspberries stored with either SO_2 generator tended to have lower pH and higher acidity than control raspberries on a given storage date (Table 2).

All of the color parameters measured were affected by SO_2 treatment (Table 1 and 2). SO_2 treated raspberries

Table 1—Main effects of sulfur dioxide treatment, storage temperature, and storage time on red raspberry quality, 1981[a]

Main effect	Mold: Howard Mold Count (%)	Mold: Visual (%)	Sol./sol. (%)	pH	Acidity (%)	Total anthocyanins (Absorbance/g of fruit)	Hunter Color Difference Meter: L	a	b
SO_2 treatment									
Open control	91a	39b	10.6a	3.46a	1.49c	34.2a	20.4d	27.8c	9.2d
Closed control	89a	45a	9.5c	3.40b	1.41d	30.1b	21.7c	27.5c	9.9c
Fast release	83b	23c	10.1b	3.27c	1.64b	28.9bc	23.3b	31.7b	10.4b
Dual release	76c	0d	10.6a	3.17d	1.75a	28.5c	25.8a	34.7a	11.3a
Storage temperature (°C)									
4	77c	0c	11.2a	3.18c	1.75a	31.2b	24.4a	23.1b	11.8a
14	86b	29b	10.6b	3.30b	1.55b	34.1a	22.2b	32.1c	10.2b
25	91a	52a	8.9c	3.48a	1.41c	25.9c	21.7c	36.1a	8.6c
Storage time (days)									
3	78b	4c	11.0a	3.23b	1.76a	32.8a	23.9a	35.1a	11.3a
6	86a	33b	10.5b	3.19c	1.63b	32.3a	22.7b	31.4b	10.1b
12	90a	43a	9.2c	3.55a	1.32c	26.2b	21.7c	24.8c	9.3c

[a] Means within columns within main effects pooled across three replications and all other variables. Values in a column followed by a common letter are not significantly different at $P < 0.05$ by Duncan's Multiple Range Test.

Table 2—Interactive effects of sulfur dioxide treatment x storage time on red raspberry quality, 1981[a]

SO_2 treatment	Time (d)	Mold: Howard Mold Count (%)	Mold: Visual (%)	Sol./sol. (%)	pH	Acidity (%)	Total anthocyanins (Absorbance/g of fruit)	Hunter Color Difference Meter: L	a	b
Initial		48	0	11.6	3.00	1.92	23.9	26.5	36.8	12.6
Open control										
	3	90abc	7de	11.4a	3.26cd	1.82abc	36.3a	22.7d	34.1cd	10.9c
	6	89a-d	50c	11.0ab	3.28c	1.66d	33.4bc	20.2g	29.1e	9.1g
	12	93ab	59b	9.5de	3.83a	1.19f	29.8ef	18.4h	20.2g	7.7h
Closed control										
	3	82cde	9d	11.0ab	3.24cde	1.79bc	34.6a	23.2d	34.6bc	11.1bc
	6	91ab	60b	9.7c	3.20ef	1.56e	31.6cde	21.1f	28.3e	9.5ef
	12	95a	67a	7.8f	3.78b	1.05g	23.6h	20.4g	19.5g	9.2fg
Fast release										
	3	70f	1e	10.9ab	3.21ef	1.88a	31.2de	24.6c	35.6ab	11.4ab
	6	84b-e	23d	10.3c	3.21ef	1.76c	33.6b	23.2d	33.3d	10.3d
	12	94a	44c	9.1e	33.9c	1.50e	23.9h	22.0e	26.3f	9.6e
Dual release										
	3	70f	0f	10.9ab	3.19f	1.86ab	28.9fg	25.3bc	35.9a	11.6a
	6	80de	0f	10.8bc	3.09g	1.83abc	32.8bcd	26.3a	34.9abc	11.4ab
	12	78ef	0f	10.3c	3.22de	1.79bc	27.3g	25.7ab	33.2d	10.8c

[a] Means within columns pooled across three temperatures and three replications. Values in a column followed by a common letter are not significantly different $P < 0.05$ by Duncan's Multiple Range Test.

tended to have lower TAcy concentrations and were lighter (higher CDM "L"), redder (higher CDM "a"), and less blue in color (higher CDM "b") than control fruits (Table 1). TAcy concentration increased during 3 days storage with the greatest increase occurring in control fruits (Table 2). The relatively high TAcy concentrations for the open control fruit were partially due to dehydration of the raspberries which resulted in concentration of the pigment. CDM "L", "a", and "b" values dropped more rapidly in both sets of control fruit then either SO_2 treatment during storage (Table 2). Bleaching of anthocyanins by SO_2 was observed at both 14 and 25°C, while the fruit stored at 4°C with SO_2 had a lustrous red color. During storage the color of control fruits shifted from a bright red color to a dull reddish purple color as indicated by decreases in CDM "L," "a," and "b". This shift in color was probably due to the shift in ionic form of the anthocyanin pigments due to the increase in fruit pH during storage.

1982 Studies

In 1982 fresh-market raspberry quality and visual mold responded to SO_2 treatment, storage temperature, and storage time in a similar manner as 1981 (Table 3). However, HMC was not affected by SO_2 treatment in 1982. During storage mold concentration and raspberry quality of the two maturities changed in a similar manner within a given SO_2 treatment. Therefore, these data are not presented.

Soluble solids, pH, and TAcy concentration did not differ between the two maturities (Table 3). Acidity of fresh-market raspberries was higher than the processing raspberries. Processing raspberries were darker (lower CDM "L"), less red (lower CDM "a") and bluer (lower CDM "b") in color than fresh market raspberries. Raspberries stored with either of the SO_2 generators were visually lighter red in color than the controls and some irregular bleaching of color from SO_2 treated fruit occurred.

SO_2 concentration in the fresh-market fruit was assayed in 1982. Fruit stored with the FRG had reached maximum SO_2 concentrations by the third day of storage at both 4 and 14°C (70 and 230 ppm, respectively). After 12 days of storage at 4°C, the SO_2 concentration had dropped to 30 ppm while fruit stored at 14°C had SO_2 concentrations of 60 ppm. When the DRG was used at 4°C, SO_2 concentration decreased after 3 days of storage from 120 on day 3 to 105 ppm on day 12 of storage. Conversely, when DRG were used at 14°C, SO_2 concentration steadily increased from 120 to 365 ppm through the 12 days of storage.

DISCUSSION

VISUAL OBSERVATIONS of mold indicated that the use of SO_2 generators especially DRG reduced mold growth. However, Howard mold count levels did not support these observations. The influence of SO_2 on fruit quality was temperature dependent. Fruit stored with SO_2 at room or an elevated temperature had irregular bleaching of drupelets. When used at a refrigerated temperature the SO_2 treated fruit had a desirably brighter red color. Even if SO_2 generators consistently maintained lower HMC levels in raspberries, the generators would not be suitable for use in fresh market products due to SO_2 residues in the fruit and the potential for significant quality reduction. Lower SO_2 concentrations might reduce SO_2 injury to raspberry fruits; however, mold was not controlled by the levels used in this study. Use of the SO_2 generators in raspberries for the processed market should be limited to products receiving a heat treatment to remove a majority of the SO_2 residue. Cruess and El Nouty (1927) reported that vacuum boiling at 70°C or open kettle boiling at 100°C of pitted cherries reduced SO_2 concentrations from 720 to less than 25 mg/kg of fruit within 20 min. Use of SO_2 generating pads as a source of SO_2 for processing raspberries might not be feasible due to the expense of materials and handling. Other SO_2 sources such as compressed SO_4 gas, sulfurous acid $CaSO_4$, and $K_2S_2O_5$, have been shown to be effective in preserving fruits (Atkinson and Srachan, 1964; Cruess, 1933; Cruess and El Nouty, 1927).

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Table 3—Main effects of SO_2 treatment, fruit maturity and storage time at 14°C on red raspberry quality, 1982[a]

Main effect	Mold: Howard Mold Count (%)	Mold: Visual (%)	Sol./sol. (%)	pH	Acidity (%)	Total anthocyanins (Absorbance/g of fruit)	Hunter Color Difference Meter L	a	b
SO_2 treatment									
Open control	78a	52b	10.7a	3.48a	1.36b	36.6a	18.8d	25.0c	8.1d
Closed control	85a	71a	8.9c	3.39b	1.23c	28.4b	19.9c	23.4d	8.7c
Fast release	76a	20c	10.1ab	3.22c	1.58a	30.8b	21.2b	32.1b	9.3b
Dual release	81a	3d	9.9b	3.14d	1.63a	30.4b	24.0a	34.3a	10.1a
Maturity									
Fresh	83a	29b	10.0a	3.29a	1.54a	31.4a	21.8a	30.2a	9.3a
Processing	76b	43a	9.8a	3.32a	1.37b	31.7a	20.1b	27.2b	8.8b
Days at 14°C									
3	60c	5c	10.2a	3.15c	1.59a	35.0b	21.3a	33.2a	10.0a
6	82b	39b	9.9a	3.25b	1.48b	38.8a	20.2b	29.3b	9.0b
12	98a	66a	9.6a	3.53a	1.29c	20.9c	21.4a	23.7c	8.1c

[a] Mean within columns within main effects pooled across three replications and all other variables. Values in a column followed by a common letter are not significantly different at $P < 0.05$ by Duncan's Multiple Range Test.

Effect of Sorghum Variety on Baking Properties of U.S. Conventional Bread, Egyptian Pita "Balady" Bread and Cookies

M. M. MORAD, C. A. DOHERTY, and L. W. ROONEY

ABSTRACT

Five genetically different sorghums were ground and partially substituted for bread and cookie flour in U.S. white pan bread, Egyptian "Balady" bread and sugar cookies. Except for brown sorghum, water absorption, peak time, stability and time to breakdown, decreased as sorghum increased in the formula. Brown sorghum produced a better quality dough and higher bread volume compared to other sorghums. Bread volume decreased with increasing level of sorghum substitution. Crumb and crust color were directly related to sorghum variety and level of substitution. Replacement of bread flour with up to 30% ground sorghum produced acceptable Egyptian "Balady" bread. Spread factor of sugar cookies increased with increasing levels of sorghum.

INTRODUCTION

SORGHUM (*Sorghum bicolor* [L.] Moench) is the world's fifth leading cereal. Western societies use it only as an animal feed. However, in many African, Asian and Latin American countries, sorghum is used for human consumption and is utilized in porridges and both leavened and unleavened bread. In addition, sorghum is incorporated in snacks and is popped, chewed and malted (Prasada Rao and Murty, 1982). Several studies have indicated the possibility of incorporating sorghum in wheat flour at various levels when wheat is in short supply. Such composite flours can be used for producing bread, biscuits and other snacks (Badi et al., 1976; Rooney et al., 1970; Hulse et al., 1980). Perton (1977) reported the satisfactory production of French-type bread from composites of 85% wheat flour (87% extraction) and 15% sorghum flour. Bhatia et al. (1968) substituted 20% of wheat flour with sorghum flour for breadmaking. He reported the bread acceptable and the calculated protein content comparable to bread made from 100% wheat. Also, lipids, mineral and fiber contents of the composite were higher than bread made from 100% wheat flour. Sumner and Nielsen (1976) produced acceptable Nigerian bread using an 80/20 wheat/sorghum composite flour blend. When compared with 100% wheat flour bread, 80/20 wheat/sorghum bread had darker brown external and internal color, increased coarseness and firmness and stronger cereal flavor. Awadalla (1974) reported using 20% whole or decorticated millet flour with 80% Dutch wheat flour of 100%, 80% and 70% extraction rate in Dutch pan bread and Egyptian flat bread. The baking quality was impaired in both breads as judged by loaf volume and internal and external characteristics, but was improved by the addition of dough conditioners ($KBrO_3$, fat and calcium stearoyl lactylate). The sorghum flour had the greatest effect on the quality of the bread with 80% extraction wheat flour.

The U.S. Marketing Classes for sorghum are white, yellow, brown and mixed. Those clases separate sorghums by color and presence or absence of a pigmented testa. The objectives of this study were to determine the effect of sorghum variety on chemical composition and functional properties of wheat flour-sorghum blends and to partially substitute wheat flour with whole ground sorghum in U.S. conventional bread, Egyptian balady pocket bread and sugar cookies.

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MATERIALS & METHODS

Wheat and sorghum samples

Five sorghum varieties representing brown (Tx2566 and ATx 623xSC0103-12, white (77CS5) and yellow (ATx399xRTx430) market classes and a white, waxy endosperm type (BTx615) were used. Characteristics and genetic descriptions of the varieties were reported by Doherty et al. (1983). All sorghums were milled with a Udy Laboratory Mill (Udy Corp., Fort Collins, CO) through a 1 mm screen mesh. A hard red spring wheat flour (CS-79) was obtained from the US Grain Marketing Research Laboratory (Manhattan, KS). Extraction rate of the wheat flour was 72%. The flour was used for making white pan-bread and Egyptian pocket bread. A commercial cookie flour was used for the preparation of sugar cookies. Chemical composition of the flours is given in Table 1.

Chemical analyses

Crude protein was determined using a modified micro-Kjeldahl method (Technicon Industrial Method 369-75A/A$^+$). Starch was measured by an automated method of analysis (Technicon Industrial Method SFA-0046FAB). Tannins were determined using the modified Vanillin-HCl method (Maxson and Rooney, 1972). Phytate (Phytate-P) and phosphorous (Total-P) were determined by the method of Doherty et al. (1982). Color was evaluated with a Gardner Color Difference Meter (Gardner Laboratories, Bethesda, MD). A standard tile of L = 77.3, a = −1.7 and b = 22.8 was used as a reference. The "L" value represents the degree of lightness, the "a" value denoted redness or greenness, and the "b" value measures the yellowness or blueness.

Rheological properties

Mixograms (10g) were prepared using the procedure of Finney and Shogren (1972). Farinograph (50g) was performed according to the AACC (1976) Method.

Baking performance

U.S. pan white bread. Bread was prepared using a straight dough procedure described by Finney (1977). The formula consisted of 100g total flour (14% mb), 2.2g dry bakers yeast (Fermipan), 1.5g salt, 6g sugar, 0.25g diastatic malt (54 Du/g, 20°C), 3g vegetable shortening (Crisco) optimum amount of water (obtained from preliminary farinographs) and 50 ppm ascorbic acid. Total fermentation time was 90 min and proofing time was 30-35 min. The bread was baked for 24 min at 216°C. Volume was measured by rapeseed displacement.

Pita "Balady" bread. The Egyptian pita "Balady" bread was prepared using a formula consisting of 100g total flour, 0.5g dry yeast (Fermipan), 1g salt, 3g sugar and 70-75 mL of water. The dough was fermented for 75 min then shaped in a round flat form using a pin roller (thickness of 0.5 cm and diameter of 15-20 cm). The dough was left for 15 min proofing time, then baked at 302°C for 5 min. Pocket formation was evaluated subjectively by the authors immediately after the bread is removed from the oven. Excellent puffing is the complete separation of the two crust layers. Satisfactory puffing is the separation of the two crust layers except for some parts of the loaf. The bread that did not puff was rated poor.

Sugar cookies. Sugar cookies were prepared using a commercial

cookie flour and ground sorghum blends according to the AACC (1976) procedure. The formula consisted of 225g flour, 130g sugar, 64g vegetable shortening (Crisco), 2.1g salt, 2.5g baking soda, 25 mL distilled water and 33 mL 8% dextrose solution. Spread factor was determined according to the AACC (1976) method.

RESULTS & DISCUSSION

Chemical composition of sorghum varieties

The sorghum samples were similar in protein and starch contents (Table 1). Color varied between varieties with the white, waxy variety (BTx615) being lightest in color and the brown varieties (Tx2566, ATx623xSC0103-*12*) the darkest. Tannin levels were highest in the brown sorghums while the others contained only trace levels (<0.1%). Eggum et al. (1981) found the nutritional value of whole white sorghum grain was comparable to other cereals based on protein and energy availability. On the other hand, diets containing high tannin sorghums produced low growth rates for rats (Maxson et al., 1973). The percentage of phytate-phosphorus of total-P in ground sorghum was higher than that of wheat flour (Table 1). This is reasonable since the pericarp aleurone area, a high phytate area, had not been removed. Although phytases are actively present in wheat, their activities have not been documented in sorghum.

Rheological properties

Rheological properties measured by mixograph and farinograph are shown in Table 2. Except for the brown sorghums, replacing wheat flour with 0, 10, 20 and 30% of sorghum adversely affected dough properties. As the sorghum level increased, water absorption, peak time, time to breakdown and stability decreased while mixing time increased. The brown varieties (ATx623xSC0103-*12* and Tx2566) had the best dough properties including stability, peak time and time to breakdown of the wheat/sorghum flour blends. The deterioration in the rheological properties was primarily due to the dilution of wheat gluten upon sorghum substitution. In the brown varieties the effect of diluting the wheat gluten was less than the other wheat/sorghum flour blends, as indicated by better dough formation, stability and higher loaf volumes (Table 2). The effect

Table 1—Chemical composition of ground sorghum and wheat flours on a dry weight basis

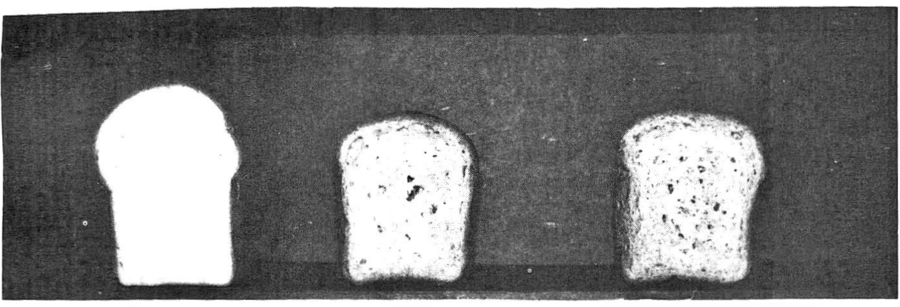
Variety	Starch %	Protein[a] %	Phytate-P of Total-P %	Color[b]	Tannin[c]
Tx2566 (Brown	69.5	11.8	70.9	67.0	0.24
ATx623xSC0103-12 (Brown)	70.7	11.7	69.8	68.5	2.12
ATx399xRTx430 (Red)	71.4	12.1	68.1	74.4	Trace
77CS5 (White)	74.6	11.6	63.7	74.5	Trace
BTx615 (White, Waxy)	76.3	11.7	71.2	78.6	Trace
Whole Wheat Flour (Commercial)	67.8	14.7	58.5	86.5	Trace
Bread Flour (CS-79) 72% extraction	81.3	12.9	22.5	98.8	Trace
Cookie Flour	83.3	10.4	19.5	98.4	Trace

[a] Nx6.25 for sorghum, Nx5.7 for wheat flour.
[b] Major differences in color observed between samples were in the "L" value std tile: L = 77.3, a = —1.7, b = 22.8.
[c] Expressed as catechin equivalents/100 mg sample.

Table 2—Rheological properties of doughs prepared from wheat/sorghum flour blends

Variety	Water[a] absorption %	Mixing[b] time (Min)	Peak[a] time (Min)	Stability[a] (Min)	Time to[a] breakdown (Min)	Loaf volume (cc)
Wheat flour						
72% Extraction	64.2	5:25	11:00	17:30	18:00	950
Tx2566 (Brown)						
10%*	61.6	5:40	12:00	17:30	14:00	820
20%*	59.4	6:00	12:00	16:30	15:00	700
30%*	57.8	6:15	12:00	15:30	16:00	645
ATx623xSC0103-12 (Brown)						
10%	61.4	5:30	12:30	18:00	16:30	835
20%	59.0	6:15	14:00	20:30	17:00	760
30%	57.8	6:25	17:00	24:00	18:30	665
A399xRTx430 (Red)						
10%	61.6	5:30	10:30	14:30	14:00	815
20%	59.2	5:50	9:00	11:30	11:00	735
30%	57.6	6:00	7:30	10:15	9:30	625
77CS5 (White)						
10%	61.4	5:30	10:30	14:30	13:40	795
20%	59.3	5:50	9:00	13:30	11:30	665
30%	57.8	6:15	7:30	11:40	10:30	590
BTx615 (White)						
10%	61.4	5:30	10:00	15:15	14:00	785
20%	59.3	5:30	9:00	14:30	12:30	710
30%	57.7	5:30	9:00	12:15	12:30	615

[a] From the Farinograph.
[b] From the Mixograph.
* Indicates percent of wheat flour replaced by sorghum flour.



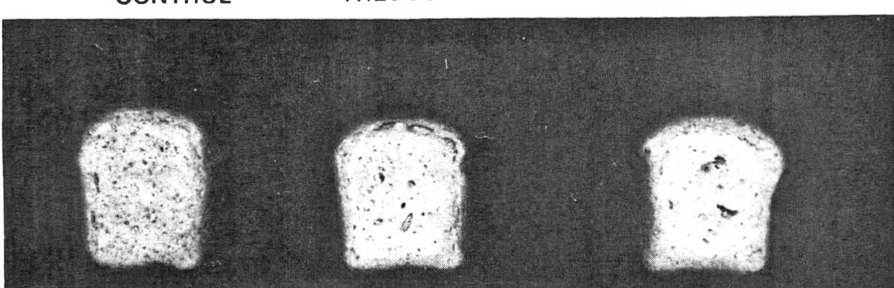


Fig. 1—Internal appearance of pan bread made from wheat flour replaced with 20% ground sorghum from 5 different varieties.

Fig. 2—External appearance of Egyptian pita "Balady" bread made from white flour replaced with 30% ground sorghum from the following varieties: (1) Tx2566 (brown sorghum); (2) ATx623x SC0103-12 (brown sorghum); (3) ATx399xRTx430 (red sorghum); (4) 77CS5 (white sorghum); (5) BTx615 (white, waxy sorghum).

was more pronounced with the ATx623xSC0103-*12* variety which has the highest tannin content (Table 1). As tannins are known to bind with protein (Hulse, 1979), a tannin-gluten complex is likely to be formed. This complex might be responsible for the change in rheological properties. More work is required to determine the effects of sorghum tannins on dough properties to clarify the observed differences.

White pan and Egyptian pita "Balady" breads

Loaf volume of white pan bread decreased significantly as the amount of sorghum increased in the formula (Table 2 and Fig. 1). A decrease in loaf volume of 100 mL for each 10% of sorghum added was generally observed. Although the differences in loaf volume between varieties were relatively small, the brown variety (ATx623xSC0103-*12*) produced the largest volume. The rheological properties of the same variety as indicated before was also the best. Crumb and crust color was directly related to the original color of sorghum.

Egyptian pita "Balady" bread is a flat, circular loaf consisting of two layers. The bread is produced in Egypt using 82-95% extraction wheat flour depending on the national economy and the price of wheat. Although there is no specific characteristic to judge the quality of pita bread, puffing formation, ease of layer separation, crust, shape and color are the most important parameters. Pocket formation for all varieties was excellent when sorghum replaced up to 30% of wheat flour (Fig. 2 and Table 3). At 40 and 50% substitution, the pocket formation was rated satisfactory to poor in most varieties. Also at the higher levels, white and white waxy varieties were slightly better than the other varieties.

The color was directly affected by both sorghum variety and replacement (Table 3). In general, a white color is not required for this bread, but is preferred. The color value for pocket bread made from whole wheat flour was similar to those made from brown and yellow sorghum at 30% replacement and white sorghum up to 40% replacement. White, waxy sorghum at 50% replacement was lighter than the whole wheat flour bread. This is important since high extraction flour is mainly used in baking Egyptian Pita "Balady" bread.

Quality of sugar cookies

Supplementation of cookie flour with up to 50% sorghum increased the spread factor of sugar cookies (Table

4). The increase was more pronounced at 40 and 50% replacement. Sugar cookies made from 100% sorghum had the highest spread factor for all varieties. Increased spread factor was due in part to differences in particle size between the cookie flour and ground sorghum. As the level of sorghum substitution increased, wheat protein was diluted. Starch-protein-water interactions were not strong enough to support the larger particle size, producing a cookie with a larger spread factor. Lipids are also important in obtaining cookie spread (Cole et al., 1960; Kissel et al., 1971). Sorghum contains approximately 3.5% lipids (Rooney, 1978). Lipid content in the cookie formula would increase with the amount of sorghum substitution producing cookies with a higher spread ratio. This factor would be most evident in the 100% sorghum cookies. Badi and Hoseney (1976) reported cookies made from 100% sorghum flour (60% extraction) did not spread during baking. Sorghum flour at 60% extraction would contain less lipids due to the removal of the germ during milling. Smaller particle size and difference in formulation would contribute to reduced cookie spread. Color was directly affected by both amount and type of sorghum used to replace wheat flour (Table 5 and Fig. 3). At 10% sorghum replacement, cookie color was relatively similar to the control except with the brown variety, ATx623xSC0103-*12*, which was the darkest. As sorghum replacement level increased in the formula, 'L' value "degree of lightness" of the cookies decreased in all varieties. Cookies made from 100% ground sorghum varied in color. The brown variety produced cookies darker in color compared to the other varieties. Another study indicated molasses cookies substituted with up to 50% dried distillers grains obtained from white sorghums were acceptable to consumer and comparable to the control (Morad et al., 1983).

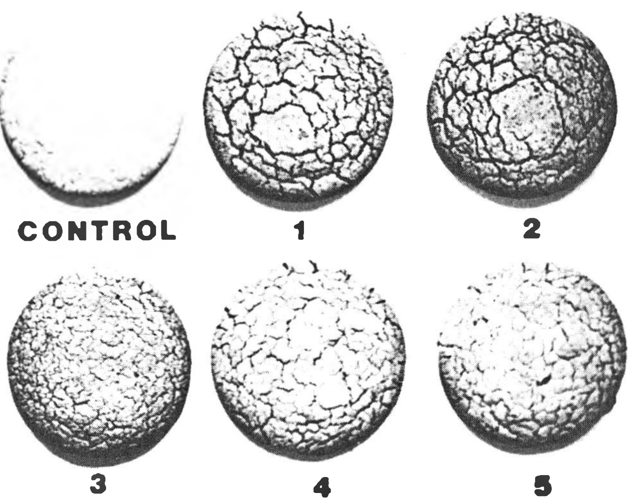


Fig. 3—External appearance of sugar cookies replaced by 30% of ground sorghum from the following varieties: (1) Tx2566 (brown sorghum); (2) ATx623xSC0103-12 (brown sorghum); (3) ATx399x RTx430 (red sorghum); (4) 77CS5 (white sorghum); (5) BTx615 (white, waxy sorghum).

Table 3—Color ("L" value) and pocket formation of egyptian pita "Balady" bread made from wheat/sorghum flour blends

	Pocket formation					Color "L" value[a]				
	10%	20%	30%	40%	50%	10%	20%	30%	40%	50%
TX2566 (Brown)	E[b]	E	E	S	P	60.0	50.4	48.0	42.1	41.9
ATx623xSC0103-12 (Brown)	E	E	S	P	P	58.1	51.2	48.4	42.4	39.7
ZTx399xRTx430 (Red)	E	E	E	S	P	60.6	53.5	59.9	47.2	43.9
77CS5 (White)	E	E	E	S	S	63.2	58.4	54.7	49.4	45.8
BTx615 (White)	E	E	E	E	S	67.1	61.4	57.4	53.8	52.8
72% Extraction flour			E				68.9			
Whole Wheat flour			E				48.2			

[a] Major differences in color observed between samples were in the "L" value, std tile: L = 77.3, a = —1.7, b = 22.8.
[b] E = Excellent; S = Satisfactory; P = Poor.

Table 4—Spread factor of sugar cookies

	Sorghum replacement %						
Variety	0	10	20	30	40	50	100
Tx2566 (Brown)	77.6	78.9	77.9	85.6	86.3	89.6	130.8
ATx623xSC0103-12 (Brown)	77.6	80.3	78.3	77.8	81.4	94.2	108.82
ATx399xRTx430 (Red)	77.6	79.4	78.6	76.8	75.4	71.2	68.49
77CS5 (White)	77.6	76.8	79.2	87.0	86.2	88.9	117.11
BTx615 (White)	77.6	80.5	74.8	78.3	78.8	77.8	91.0

Table 5—Color ("L" value) of sugar cookies[a]

	Sorghum replacement %						
Variety	0	10	20	30	40	50	100
Tx2566 (Brown)	68.6	63.9	58.6	58.0	53.5	53.5	42.5
ATx623xSC0103-12 (Brown)	68.6	61.9	60.4	57.6	54.7	52.4	46.0
ATx399xRTx430 (Red)	68.6	64.9	60.8	59.8	57.7	56.2	51.0
77CS5 (White)	68.6	65.0	63.5	60.6	59.0	58.3	48.6
BTx615 (White)	68.6	63.7	65.0	62.6	59.8	59.8	55.6

[a] Major differences in color observed between samples were in the "L" value, std. tile: L = 77.3, a = —1.7, b = 22.8.

CONCLUSIONS

WHOLE, GROUND SORGHUM was successfully used as a partial replacement for wheat flour in white pan bread, pita bread and sugar cookies. Substitution of hard wheat flour with up to 20% sorghum decreased the loaf volume of pan bread without affecting the taste. Bread crumb color, especially those made with brown sorghums, could compete with bread made from whole wheat. Loaf volumes of breads partially substituted with brown sorghums were higher than those replaced with white or yellow sorghums. The role of tannins in breadmaking, particularly their interaction with wheat gluten, should be further investigated. An excellent pita bread was produced from bread flour replaced by 30% of the ground sorghum. Brown-colored sorghums at 30% substitution were similar to the whole wheat control. This is an advantage since high extraction flour is usually used in Egypt and other countries in making pita bread. With the agronomic advantages of sorghum over wheat and maize, those countries would be able to reduce their total dependence on imported wheat. Incorporation of sorghum into cookie flour increased the spread factor of sugar cookies. Color was the major difference observed between cookies made from the five sorghum varieties.

In general, the extreme color ranges of wheat/sorghum blends suggest their applicability in other type cookies (molasses, chocolate, oatmeal) and variety breads where a dark color is desired.

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Solubilization of the Red Beet Cell Wall Betanin Decolorizing Enzyme

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ABSTRACT

The red beet root cell wall betanin decolorizing enzyme was solubilized by digestion of tissue slices with cellulase, pectinase and glusulase. The cell wall hydrogen peroxide generating system was inactivated by the digestion process. Lysis of released protoplasts demonstrated the presence of a soluble intracellular decolorizing enzyme. The intracellular form, representing approximately 25% of the total activity, appears to have become ionically bound to cell wall fragments when intact tissue was homogenized and could be released by washing with 1M NaCl.

INTRODUCTION

PLANT PEROXIDASES are thought to play an important role in processes such as lignification (Egley et al., 1983; Halliwell, 1978; Harkin and Obst, 1973), senescence (Frenkel, 1972; Haard, 1973) and off flavor formation (Burnette, 1977). Peroxidases are found both intracellularly and bound to cell walls [See Wang and Luh (1983) and Gkinis and Fennema (1978) for references]. Cell wall peroxidases can be classified as either ionically or covalently bound. Ionically bound activity is generally defined as the activity released from isolated cell walls upon washing with high ionic strength salt solutions. Covalently bound activity is considered activity remaining with cell walls following the salt wash. It has been possible to solubilize covalently bound cell wall peroxidases from isolated cell walls by treatment with cell wall-degrading enzymes (Strand et al., 1976) or more directly, by dissolving tissue slices with protoplast releasing enzymes (Yung and Northcote, 1975). Enzymatic cell wall enzyme release has led to the suggestion that some ionically bound cell wall enzymes are intracellular *in vivo* and become wall-bound when intact tissue is disrupted by harsh methods such as homogenization (Yung and Northcote, 1975).

The objective of this study was to solubilize the betanin decolorizing enzyme found in red beet storage tissue. The enzyme, which appears to be a peroxidase (Wasserman and Guilfoy, 1983; Wasserman et al. 1984) was localized predominantly in a 0-1,000 x *g* particulate fraction (Lashley and Wiley, 1979; Soboleva et al., 1976). To achieve enzyme release, both tissue slices and isolated cell walls were incubated with the cell wall digestion enzyme mixture utilized by Schmidt and Poole (1980) for protoplast production.

MATERIALS & METHODS

Materials

Red beets (*Beta vulgaris* L.) were purchased from a local market. Tops were removed before use and roots were surface sterilized. Betanin was purified as previously described (Wasserman and Guilfoy, 1983). Pectinase and bovine serum albumin were obtained from Sigma Chemical Co. Cellulysin was purchased from Calbiochem-Behring and glusulase was supplied by Endo Laboratories (Garden City, NY). All solutions were prepared using sterilized water.

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Enzyme assays

Decolorizing activity was measured by monitoring betanin loss at 538 nm and 25°C (Wasserman and Guilfoy, 1983). Unless otherwise specified, assay mixtures contained 30 μM H_2O_2 and 100 mM citrate-phosphate buffer, pH 3.4, in a final volume of 1.0 mL. Units of activity are defined as the number of nmoles per mL of digestion mixture of betanin decolorized per min.

Solubilization

Cell wall decolorizing enzyme solubilization was performed by two methods: enzymatic digestion of intact tissue discs (Method A) and enzymatic digestion of isolated cell wall fragments (Method B).

Method A. Intact tissue discs (1g; 0.5 mm diameter by 1 mm thick) were bubbled with air over night in water and 1 mM dithioerythritol, and were then incubated with 10 mL of digestion buffer containing 0.6M sorbitol, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 1 mM KCl, 1% (w/v) BSA, 2% (w/v) cellulysin, 2% (v/v) pectinase, 2% (v/v) glusulase and 30 mM Mes-Tris, pH 5.5 (Schmidt and Poole, 1980). Digestions were performed at 30°C in a shaking water bath for various times. Protoplast release was quantitated by counting cells with a hemocytometer. Protoplasts and debris were removed by centrifugation at 100 x *g* at 4°C. The supernatants were measured for decolorizing activity as described. Background peroxidase activity from the protoplast digestion enzymes, approximately 15-25% of activity released, was subtracted. Background activity levels of each of the digestion enzymes are summarized in Table 1. Betanin leakage was quantitated by measuring the absorbance of the digestion medium at 538 nm. Intracellular decolorizing enzyme activity was measured by lysing protoplasts in 30 mM Mes-Tris buffer, pH 5.5, centrifuging away debris at 1000 x *g*, and assaying the supernatants as described.

Method B. Cell walls were isolated as follows: One gram of tissue discs was suspended in 10 mM sodium phosphate buffer, pH 6.9, and homogenized at 4°C in a Dounce homogenizer until a uniform suspension was obtained. The homogenate was centrifuged six times at 1,000 x *g*, resuspending in 10 mM phosphate buffer, pH 6.9, to remove residual pigment and other soluble contaminants. The washings (1.5 mL each) were pooled and then assayed for activity. The cell wall fragments were then treated with 1 mL of digestion buffer (Method A) for 3 hr. The solubilized enzyme was assayed as described. In some preparations, ionically-bound decolorizing enzyme was removed prior to digestion by washing cell walls twice with 1M NaCl.

Protein assay

Protein was measured by the TCA precipitation method of Peterson (1977).

RESULTS

THE TIME-COURSES of decolorizing enzyme release and related parameters are shown in Fig. 1. The enzyme was released into the protoplast digestion mixture within the first hour of incubation. Protoplast release was more gradual, with maximal cell counts achieved between 3 and 6 hr (Fig. 1B). After 3 hr betanin began to accumulate (Fig. 1C) indicating that cells were beginning to lyse. Although betanin leakage accelerated after 5 hr, cell counts did not decline until after 6 hr of incubation, the time when tissue dissolution was generally complete.

Table 1—Protein-binding and betanin decolorizing activity of cell wall digestion enzymes[a]

Component	Protein (mg/mL)	Protein remaining after 5 min incubation with beet discs (1.0g) (mg/mL)	Protein binding (%)	Decolorizing activity (nmol/min/mL)
BSA	9.8	8.5	13	0
Pectinase	0.3	0.3	0	1.0
Glusulase	0.8	0.7	23	0
Cellulysin	4.5	3.8	16	2.8
All	15.9	14.6	9	3.8

[a] Digestion buffers were prepared as described in Materials & Methods. Tissue discs were matted dry with a paper towel before addition to enzyme solutions to avoid dilution effects.

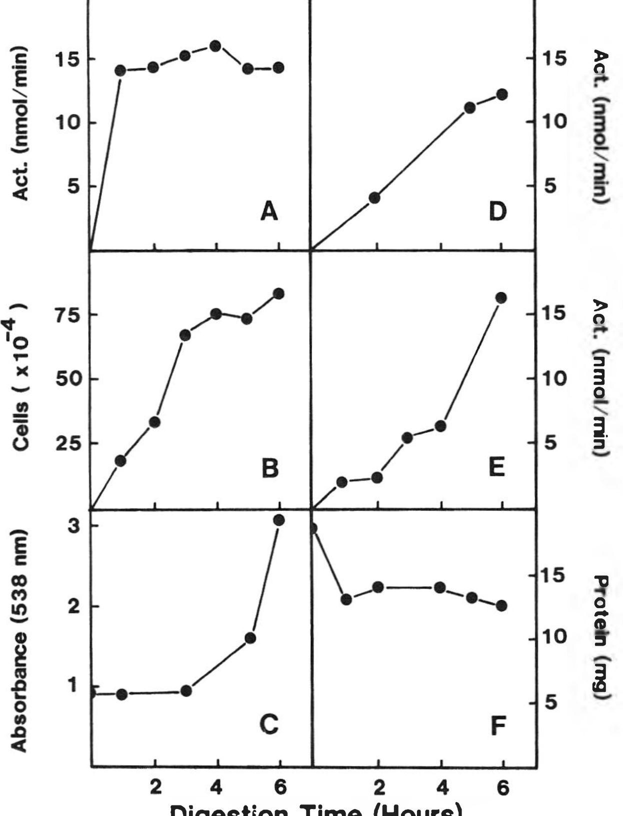

Fig. 1—Kinetics of cell wall solubilization. Panels: (A) Decolorizing enzyme release; (B) Protoplast count; (C) Betanin leakage; (D) Decolorizing enzyme release from tissue discs incubated without enzymes; (E) Decolorizing enzyme release from lysed protoplasts; (F) Protein. Individual digestions were carried out for each time point (Method A; Materials & Methods). These data are from one of two experiments, conducted identically using different beets.

In a series of controls performed in tandem, where enzymes were omitted from digestion mixtures, peroxidase release did occur, however at a slower rate than with the enzyme-treated discs (Fig. 1D). No protoplasts were generated in control incubations.

To determine whether red beet root contains intracellular betanin decolorizing activity, released protoplasts from each sample were isolated by centrifugation at 100 x *g* and then lysed by resuspension in a hypoosmotic buffer. Fig. 1E shows that activity is released upon lysis, and that the increase in activity generally correlated with the increase in cell count. In this experiment, intracellular activity represented 31 and 51% of total activity at 4 and 6 hr, respectively. To determine whether the intracellular enzyme was membrane-bound, the 100 x *g* supernatant was centrifuged at 100,000 x *g*. No activity was found in the resuspended membranes.

The protein content of digestion mixtures during solubilization was also measured (Fig. 1F). On the addition of tissue discs to the digestion buffer, a decline in the protein content of the digestion mixture occurred. The cell walls of at least one plant, oat seedlings have been shown to have a high binding affinity for a variety of enzymes including pectinesterase and peroxidase (Jansen et al., 1960). To show that the initial decline may have been due to protein binding, the various protein components of the digestion buffer were incubated with tissue discs. Residual protein in the digestion buffer was measured after 5 min. The results of this experiment, summarized in Table 1, shows that small levels of BSA and proteins in the glusulase and cellulysin became bound to beet discs at pH 5.5. The pectinase did not bind.

Seasonal dependence of protoplast release

An important observation concerning these experiments is that the ability to generate protoplasts appears to be seasonal. These experiments were all performed during the summer months using freshly harvested beets. Attempts to prepare protoplasts using aged-dormant beets during the fall and winter months over three consecutive years have often proven futile. Resistance of aged tissue to cellulolytic digestion has been reported by Geballe and Galston (1982). In oat leaves this effect was shown to be due to the formation of lignin (Gabelle and Galston, 1983).

Solubilization of isolated cell walls and decolorizing on enzyme distribution

The ability of intact tissue discs to bind protein suggested that intracellular decolorizing enzyme might become cell wall bound when harsh homogenization techniques are utilized. Harris (1983) discusses the possibility that ionically bound cell wall enzymes are the result of cytoplasmic contamination. To answer this question, a dual approach was utilized. One approach was to digest tissue discs, as described in the previous section. The other approach was to digest isolated cell wall fragments and measure activity released into the digestion medium. In the latter method two samples were prepared. One was washed with buffer alone and the other with buffer and twice with a 1M NaCl solution to remove any ionically bound enzyme. Walls were then digested for 5 hr and the supernatants assayed for activity.

In the tissue disc digestion experiment (Table 2), 41.2 units of enzyme activity were recovered, with approximately 75% of the activity cell wall bound and 25% intracellular.

After digestion of wall fragments obtained from the same beet, a total of 45.7 units of activity were recovered, in good agreement with the direct digestion approach (Table 2). Of this activity, roughly 68% could be classified as covalently bound and the remainder, 32%, was ionically bound. No activity was found in the 1,500 x *g* supernatant. These data strongly suggest that the ionically bound cell wall form of the enzyme is intracellular and becomes bound to wall fragments on homogenization.

Effect of hydrogen peroxide

In a previous study, it was shown that isolated cell walls produce H_2O_2, which is then utilized by the decolorizing enzyme for decolorization (Wasserman and Guilfoy, 1983). Fig. 2 compares the effect of H_2O_2 on the cell wall-bound and solubilized enzyme. Several differences were observed, most notably that the H_2O_2 generating system is destroyed by solubilization. In contrast to the cell wall decolorizing

system, the solubilized enzyme exhibited no activity in the absence of added H_2O_2. Maximal decolorizing activity for the solubilized enzyme was obtained with 1 mM H_2O_2, which is 10-fold greater than the level needed by the wall-bound form.

DISCUSSION

PREVIOUS ATTEMPTS to solubilize the red beet cell wall betanin decolorizing enzyme by detergent and enzyme treatments were unsuccessful (Shih and Wiley, 1982; Soboleva et al., 1976). Use of the enzyme mixture reported by Schmidt and Poole (1980) for the production of red beet protoplasts was successful, with one modification: hemicellulase was found to have no effect on the rate of protoplast release. It was therefore omitted from this series of experiments.

Release of the cell wall enzyme from tissue discs occurred within the first hour of digestion (Fig. 1A) and was not coincident with protoplast generation, which continued over 4 hr (Fig. 1B). Thus it appears that partial cell wall digestion is sufficient for enzyme release.

The decolorizing enzyme from discs incubated in digestion buffer without degradative enzymes present was solubilized more slowly (Fig. 1D). Its release may have been due to the presence of endogenous cell wall degrading enzymes which may be activated under the conditions of incubation. Yung and Northcote (1975) also reported enzyme release from control tissue discs, but to a lesser extent than observed here. Since betanin leakage was not observed until 4 hr (Fig. 1C) it appears unlikely that the release of enzyme from the control discs was due to leakage of the intracellular enzyme. It is also unlikely that betanin leaked into the digestion medium was degraded by the decolorizing enzyme, since the pH of the digestion medium, 5.5, is well above the pH range of optimal decolorizing activity (Shih and Wiley, 1982).

The protein content of the digestion mixtures generally decreased (Fig. 1F). The immediate decline upon the addition of tissue discs is probably due to the binding of proteins present in the commercial enzyme preparations (Table 1). The slow decline between 1 and 6 hr may have been due to continued binding or alternatively, to proteolysis. It should be recognized, however, that the level of protein contained within the cell wall is low relative to the large quantities present in the digestion mix. It can be calculated from the data of Yi-qin et al. (1983) that the amount of covalently bound cell wall protein contained within 1g of *Lilium longiflorium* roots is 150 µg. Although this figure is low since a significant amount of cell wall material was lost as the result of numerous washings, it points out that the protein assay method which we utilized may not have the sensitivity to accurately quantitate protein release from 1g of beet root in the presence of the digestion enzymes.

In contrast to the cell wall enzyme, the solubilized peroxidase could no longer function in the absence of added H_2O_2. Cell wall fragments isolated by homogenization have the ability to generate H_2O_2 when placed in acidic environments (Wasserman and Guilfoy, 1983). The source of reducing equivalents appeared to be ionically bound to the walls. It is possible that *in vivo*, the reduced substrate is intracellular and like the intracellular decolorizing enzyme, the hydrogen donor becomes bound to cell walls upon tissue disruption. Alternatively, the enzymatic component of the H_2O_2 generating system may be located intracellularly and is therefore not released upon enzymatic digestion. A further possibility to explain the destruction of the H_2O_2 generating system is that the digestion enzymes used in the solubilization medium contain compounds that are inhibitory such as phenolics, which are known to interfere with the activity of many enzymes (Loomis and Battaile, 1966).

Table 2—Distribution of decolorizing activity in fractions obtained by tissue digestion and digestion of isolated cell walls[a]

Fraction	Decolorizing activity (nmol/min/g fresh wt)	% Total
Tissue digestion		
Cell wall	30.7	74.5
Soluble	10.5	25.5
Total	41.2	
Digestion of isolated walls		
Cell wall-Covalent	31.1	68.1
Ionic	14.6	31.9
Soluble	0	0
Total	45.7	

[a] Details of each method are described under Materials & Methods.

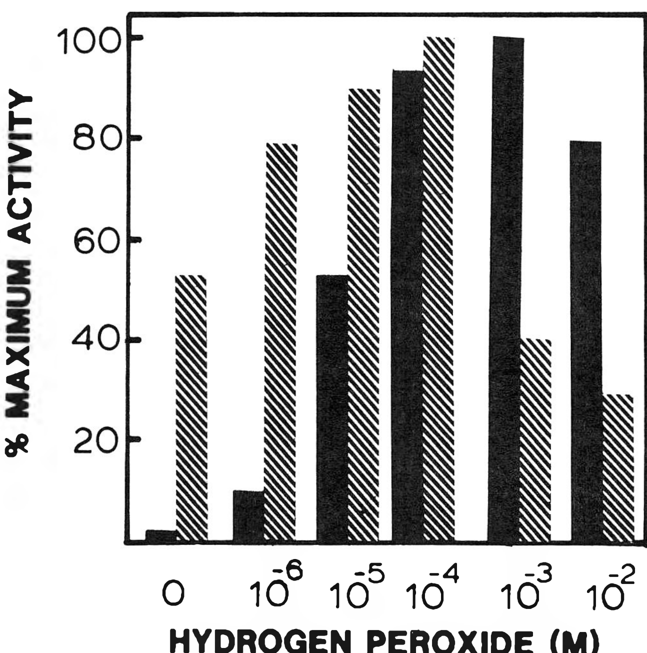

Fig. 2—Effect of hydrogen peroxide on betanin decolorizing activity by the cell wall-bound and solubilized enzymes. Solid bars: Solubilized enzyme after a 6 hr digestion. Total activity at 10^{-4}M H_2O_2 was 22.4 nmol per min per g fresh weight. Hatched bars: Cell wall activity. Cell walls were isolated and assayed as previously described (Wasserman and Guilfoy, 1983).

These results further demonstrate that gentle techniques such as tissue digestion can be utilized for solubilizing cell wall enzymes. One disadvantage of the digestion method, particularly if cell wall enzyme purification is a goal, is that large amounts of exogenous protein are present. The availability of highly purified cellulolytic and pectinolytic enzymes would greatly reduce this problem. Another possible disadvantage is that digestive enzymes may be able to cleave polysaccharide chains from glycoproteins and therefore modify the catalytic properties of cell wall enzymes.

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Inhibitory Effect of Food Preservatives on Protease Secretion by *Aeromonas hydrophila*

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ABSTRACT

Butylated hydroxyanisole (BHA), propylhydroxy parabenzoate (paraben), and sodium tripolyphosphate (TPP) were found to inhibit protease secretion by resting cells of *Aeromonas hydrophila* at lower concentrations than those required for inhibiting growth. Incorporation of the above compounds in calcium caseinate agar resulted in colonies surrounded by smaller areas of clear zones indicating inhibition of protease secretion. The results are discussed with respect to the protective influence of the above compounds against spoilage of flesh foods by microbial proteases.

INTRODUCTION

DETERIORATION in quality of flesh foods during storage is mainly due to the action of spoilage microorganisms. Several such organisms secrete proteases whose action has been reported to bring about adverse changes in freshness characteristics of meat (Herbert et al., 1971; Tarrant et al., 1973; Bala et al., 1979). Even during refrigerated storage of muscle, extracellular proteases produced by psychrotropic organisms are capable of substantial degradation of structural proteins at temperatures as low at 0°C (Porzio and Pearson, 1980; Venugopal et al., 1983). Hence although destruction of spoilage microflora is of primary importance, under conditions where this may not be feasible, restriction of the secretion of proteases by contaminant organisms would help in maintaining keeping qualities of the food. Recently, a number of food additives have been recognized to have bactericidal action. Thus butylated hydroxyanisole (BHA) and parahydroxy benzoate (paraben) have been reported to inhibit several microorganisms (Davidson and Branen, 1980; Robach, 1980; Kabara, 1981). The present communication reports on the effects of some preservatives on the secretion of extracellular protease by resting cells and on the growth of *Aeromonas hydrophila.*

MATERIALS & METHODS

Bacterial strain

The bacterium used in this study was isolated from Indian mackerel (*Rastrelliger kanagurta*) and was identified as *Aeromonas hydrophila.* The culture was maintained on plate count agar (Difco) slants. For cultivation, the organism was grown aerobically in 200 mL 0.8% (w/v) nutrient broth (Difco) on a rotary shaker. Growth of the organism was assessed by monitoring optical density of the culture in a Klett-Summerson spectrophotometer using a red filter. The culture (24 hr) was centrifuged at 5,920 x *g* for 30 min and the cell pellet was collected.

Measurement of protease secretion by the cells

Secretion of protease by resting cells of the bacterium was measured according to the method of Boethling (1975). The cells (24 hr) from 200 mL culture were washed once with 0.1M phosphate buffer, pH 7.5 and the cell pellet was suspended in 0.01M phosphate buffer, pH 7.5 to give a concentrated cell suspension (40 mL) which had a Klett reading of 500. Five mL of this suspension (Klett reading 500) were diluted with equal volume of 0.2% yeast extract (Difco) and incubated on a rotary shaker at 25°C. Aliquots (3 mL) of the cell suspension were withdrawn at regular intervals, centrifuged at 5,920 x *g* for 15 min and protease activity of the supernatant was determined. The release of protease during growth in solidified medium was monitored by plating the organism on calcium caseinate agar according to the method of Martley et al. (1970). The plates were incubated at 30°C for 48 hr. Extracellular protease activity was evidenced by the appearance of clear zones surrounding the colonies, indicative of hydrolysis of casein.

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Protease assay

The enzyme activity was determined as described by Venugopal et al. (1983). The assay system contained hemoglobin (Difco), 10 mg; 0.1M phosphate buffer pH 7.5, 0.5 mL and culture supernatant in a total vol of 2.0 mL. The mixture was incubated at 50°C for a period of 30 min. The reaction was stopped by the addition of 1 mL 20% trichloroacetic acid. After standing for 15 min, the mixture was passed through Whatman No. 1 filter paper and the tyrosine in the supernatant was determined. Unit of activity was expressed as μmol tyrosine liberated per mL culture supernatant during the incubation period.

Measurement of 1-^{14}C-DL-alanine uptake by the cells

The uptake system employed was essentially that described by Venugopal (1980). The reaction mixture contained 0.2 mL washed cell suspension (Klett reading, 500), 10 mM glucose, 10 mM DL-alanine, 10 μCi 1-^{14}C-DL-alanine (Isotope Division, BARC; Sp. activity 5.2 mCi/mmole), 0.01M phosphate buffer pH 7.5 and preservatives in a total vol of 1.0 mL. Except for TPP (1 mg), all other preservatives were incorporated at the level of 0.5 mg in the assay system. The mixture was incubated at 23°C and the reaction was started by the addition of cell suspension. At intervals aliquots (0.1 mL) of the mixture were pipetted into 10 mL chilled 0.01M phosphate buffer, pH 7.5. The diluted sample was transferred to membrane filter (0.45 μm, Millipore Corp.) and was washed with 10 mL chilled buffer. The filters were transferred to vials containing 9 mL of 1,4-dioxane containing 0.5% 2,5-diphenyl oxazole (PPO) and 10% naphthalene and were counted using a Beckman liquid scintillation counter.

Preservatives

Stock solutions (w/v) of the preservatives were prepared as follows: Butylated hydroxyanisole (BHA) (May & Baker, England), 1% in 50% ehtanol; propyl paraoxybenzoate (paraben) (Ueno pharmaceutical Co., Japan), 1% in 50% ethanol; sodium tripolyphosphate (TPP) (Amrut Laboratories, India), 6% in distilled water and sodium benzoate, 5% in distilled water. The solutions were sterilized by passing through a Millipore filter. The preservatives, at varying concentrations, were added to 50 mL sterile nutrient broth or to the cell suspension system described above to determine their effect on growth and protease secretion, respectively. The chemicals were incorporated in casein-agar plates to evaluate their effect on growth and enzyme secretion in solid media.

RESULTS

IT WAS OBSERVED that growth of the bacterium was concomitant with the appearance of the enzyme in the supernatant, and that maximum enzyme was secreted at the beginning of the stationary phase. Therefore, for studies on the effect of food preservatives on the enzyme secretion, stationary phase cells were used. Using 5 mL concentrated cells suspension (Klett reading, 500), diluted with an equal

volume of 0.2% yeast extract, enzyme release into the supernatant was found to increase during incubation up to 3 hr, after which the secretion of the enzyme decreased (Fig. 1). Hence to determine the effect of preservatives on the enzyme secretion, a maximum incubation period of 3 hr was used. The inhibitory action of various preservatives is depicted in Fig. 1. It can be seen that the enzyme secretion was inhibited to varying degrees by the food preservatives. While BHA and paraben exhibited inhibition at comparatively lower concentrations (20 µg/mL), higher levels of benzoate (1.6 mg/mL) and TPP (0.5 mg/mL) were required to cause inhibition. The effect of varying concentrations of preservatives on enzyme secretion is presented in Fig. 2. Enzyme secretion was totally inhibited at concentrations of 100 and 200 µg/mL of BHA and paraben, respectively. However, concentrations above 1 mg/mL of TPP were required for complete inhibition of enzyme secretion. At 2 mg/mL of benzoate in the assay system, only 50% inhibition of the enzyme secretion was observed.

The effect of incorporation of the preservatives in the medium on growth of the bacterium was examined. The preservatives were added to the nutrient broth at varying concentrations and growth was determined after 24 hr. The results are shown in Fig. 3. It was found that while both BHA and paraben completely inhibited growth at 100-150 µg/mL, higher levels (up to 3 mg/mL) of benzoate could not completely inhibit growth. TPP inhibited growth at 0.5 mg/mL level; however, no further inhibition was observed up to 2 mg/mL. In order to determine the effect of the preservatives on viability of the cells, the cell suspension (Klett reading, 500) was incubated for 2 hr under aseptic conditions with 1 mg each of BHA or paraben. After incubation, the cells were washed with sterile phosphate buffer (0.01M, pH 7.5), plated on nutrient agar (Difco), and survivors determined after 48 hr incubation. The cell counts/ mL obtained were: control (no preservative added), 2.5×10^{10}, BHA, 2.4×10^6, and paraben, 1.2×10^8.

The comparative inhibitory effects of the preservatives on growth and enzyme production were determined. For this purpose, 50 mL volumes of 0.8% nutrient broth, containing active culture of the bacterium inoculated at 5% level were incubated at 25°C in the presence of the preservatives. At intervals, aliquots (5 mL) were aseptically withdrawn and extent of growth and protease activity of cell-free supernatant were determined as described in Materials & Methods. The data are given in Fig. 4. It can be seen that in the absence of added preservatives, enzyme production was concomitant with growth. Incorporation of BHA, paraben or TPP at concentrations shown in the figure caused some growth inhibition; however, inhibition of protease secretion was much more pronounced. In the case of benzoate, the initial inhibition of growth and enzyme was removed on prolonged (24 hr) incubation of the culture.

The influence of preservatives on growth of and protease production by *A. hydrophila* on calcium caseinate agar plates was also examined. Release of the enzyme during growth of the bacterium was characterized by the appearance of clear zones around the colonies, resulting from the proteolysis of casein (Martley et al., 1970). Sterile solutions of the preservatives were added to petri plates containing molten medium (10 mL) and after solidification a loopful of the bacterium was spotted on the surface. After 48 hr incubation, growth and zone formation were examined. Fig. 5 shows the effect of varying concentrations of BHA on colony growth and zone formation. It can be seen that at increasing concentrations of BHA, the area of the clear zone was reduced more than the size of the colony. At a level of 0.1 mg of BHA in the plate, no clear zone was observed around the colony and at 0.15 mg BHA, only scanty growth was noticed. Similar experiments were done incorporating other preservatives in the casein agar medium. Table 1 contains qualitative data on growth and appearance

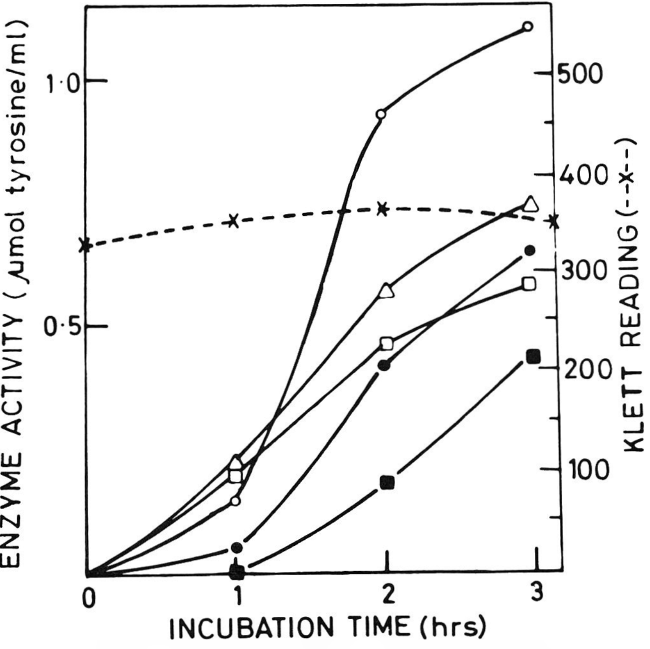

Fig. 1—Effect of various preservatives on secretion of protease by cells of Aeromonas hydrophila: x- - - -x, Klett reading of cell suspension; enzyme activity; ○——○, no preservative; △——△ benzoate, 1.6 mg per mL; ●——●, paraben, 20 µg per mL; □——□, tripolyphosphate, 0.5 mg per mL; ■——■, BHA, 20 µg per mL.

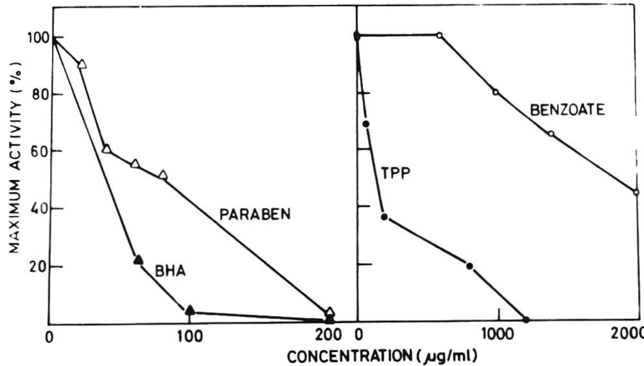

Fig. 2—Effect of varying concentrations of preservatives on protease secretion by cells of Aeromonas hydrophila. The values of protease activity are presented as percentage of control where no preservatives was added.

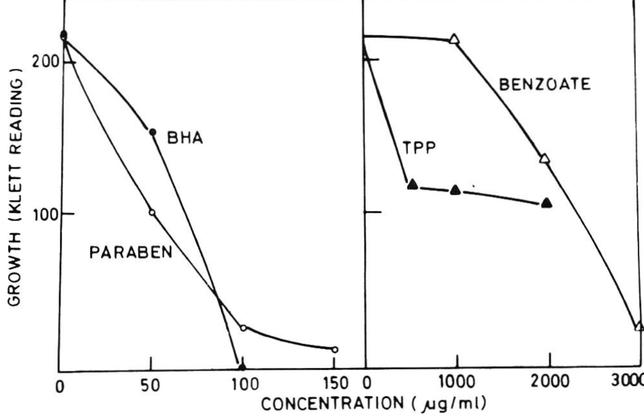

Fig. 3—Effect of varying concentrations of preservatives on 24 hr growth of Aeromonas hydrophila.

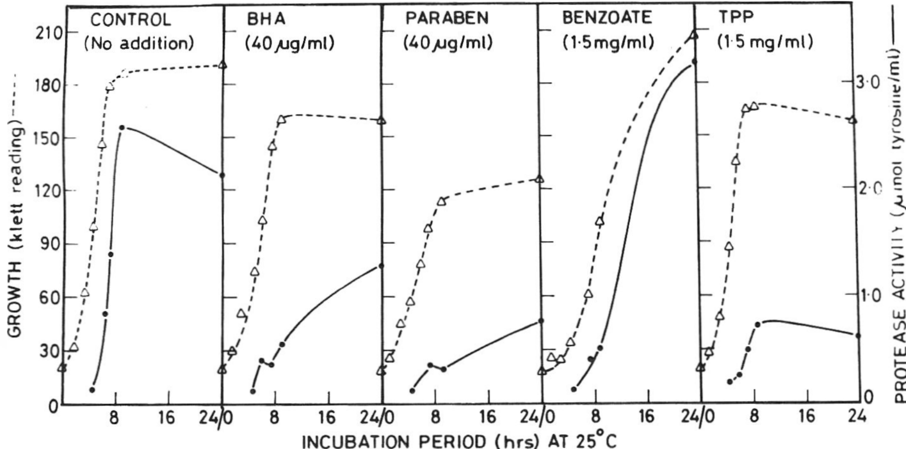

Fig. 4—Effect of preservatives on protease formation during growth of Aeromonas hydrophila.

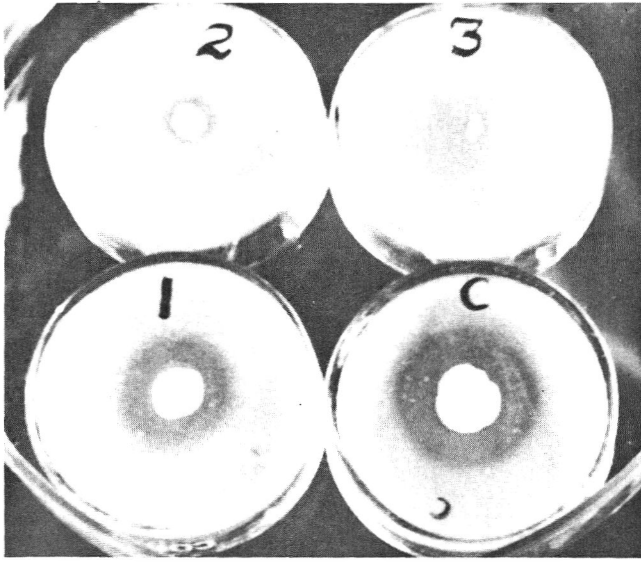

Fig. 5—Effect of BHA on growth and protease formation of Aeromonas hydrophila in calcium caseinate agar medium. The clear zone around the colony was taken as the extracellular protease activity: (C) no preservative; (1) 0.05 mg, BHA; (2) 0.1 mg BHA; (3) 0.15 mg BHA.

Table 1—Influence of varying concentrations of preservatives on growth and caseinolytic activity of A. hydrophila

Preservatives[a]	Concentration (µg)	Growth	Caseinolytic activity (area of clear zone)
No addition		+++++	+++++
BHA	50	++++	+++
	100	++	+
	150	+	–
TPP	100	++++	+++
	300	++	+
	1000	+	–
Paraben	50	++++	+++
	100	+++	++
	200	+	–

[a] Preservatives were incorporated in petri plates containing 10 mL calcium caseinate agar. The bacterium was surface plated and after 48 hr incubation at 30°C size of colonies and clear zones around the colonies were noted. +++++ = Excellent; ++++ = Very good; +++ = Good; ++ = Medium; + = Scanty; — = Nil.

of the clear zone when varying concentrations of inhibitors were present in the calcium caseinate agar plates. The results indicate that at lower concentrations of BHA, paraben and TPP the enzyme secretion was reduced, and growth was inhibited only at higher concentrations.

Effect of preservatives on alanine uptake by the cells

It is possible that the inhibitory action of the preservatives on enzyme secretion and growth is due to their ability to inhibit uptake of nutrients by the cells. We have observed that the bacterium could grow in defined medium using alanine as the sole source of nitrogen. Therefore, the uptake of alanine by the cells in the presence of the preservatives was examined. Results are presented in Fig. 6. It was observed that the compounds inhibited the uptake of the amino acid at varying levels. BHA was a more potent inhibitor than paraben, and compared equally with benzoate on inhibiting the uptake. TPP at the 1 mg level did not significantly inhibit the uptake of alanine.

DISCUSSION

THE PROTECTIVE ACTION of preservatives against spoilage of flesh foods has mainly been studied with respect to their ability to inhibit the growth of microorganisms (Davidson and Branen, 1980; Robach, 1980; Kabara, 1981). The present results show that although these compounds inhibit growth of *A. hydrophila* at certain threshold levels, the preservatives were also found to inhibit secretion of extracellular proteases by the cells at much lower levels. Involvement of microbial extracellular proteases in spoilage of flesh foods has been well recognized (Porzio and Pearson, 1980; Venugopal et al., 1983). Although storage of these foods under ideal conditions of refrigeration can minimize the proliferation of spoilage flora, the contaminating organisms present can release proteases which could lead to their spoilage. The present results on the inhibition of protease secretion by food preservatives, therefore, suggest an additional advantage of using these compounds. It is likely that the preservatives may inhibit not only the release of proteases but also other extracellular enzymes and exotoxins. Recently, BHA has been shown to inhibit growth and toxigenesis of some Aspergilli (Lin and Fung, 1983). Trimethylamine oxide reductase activity of resting cells of *E. coli* has also been observed to be inhibited by some food preservatives (Kruk and Lee, 1982).

Growth and proteolytic activity of the bacterium in broth as well as in solid medium were affected by preservatives. In both systems the secretion of the protease was inhibited at lower concentrations of the preservatives than those required to cause growth inhibition. This concurs with the report of Eklund (1980) who found that lower concentrations of propyl paraben inhibited uptake of a

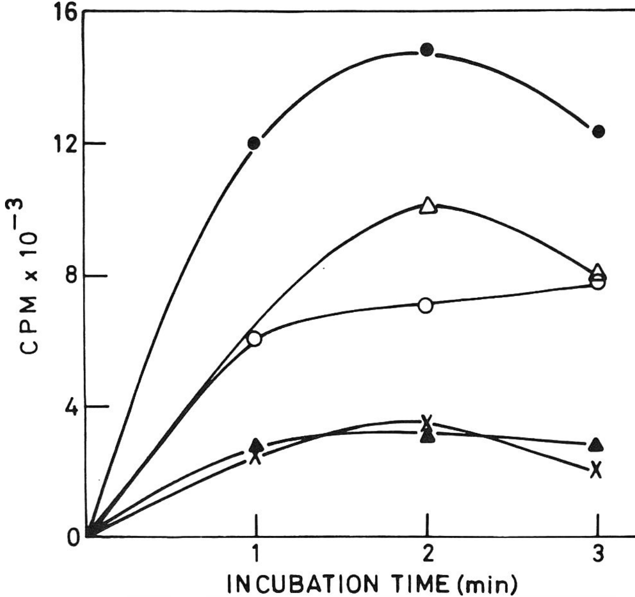

Fig. 6—Effect of preservatives on uptake of 1-^{14}C-DL-alanine by cells of Aeromonas hydrophila. The uptake system is described in the text. ●——●, no preservative; △——△, paraben, 0.5 mg; ○——○ tripolyphosphate, 1 mg; x——x, benzoate, 0.5 mg; ▲——▲ BHA, 0.5 mg.

number of amino acids by some bacteria, while higher levels of the compound were required for growth inhibition. In the present experiments, lesser amounts of BHA and paraben, as compared with benzoate and TPP, were required to inhibit enzyme secretion and bacterial growth. TPP did not completely inhibit growth. The antimicrobial action of benzoate has been recognized to be effective at lower pH range. In the present experiments the initial inhibitory effect of benzoate, on growth of and enzyme secretion by *A. hydrophila,* was not observed on prolonged incubation of the organism.

Three possible targets for antimicrobial action of food preservatives have been identified viz. cellular membrane, genetic material, and enzymes (Chichester and Tanner, 1975). Growth inhibition caused by paraben has been attributed to interference in transport of nutrients (Eklund, 1980) as well as inhibition of synthesis of RNA and DNA (Nes and Eklund, 1983). The membrane has also been recognized as the target for the action of BHA and weak organic acids (Davidson and Branen, 1980; Freese et al., 1973). In the present experiments, the preservatives used did not cause *in vitro* inhibition of the protease (data not shown). The compounds affected viability of cells as well as uptake of alanine indicating that the preservatives could impede the transport of nutrients by the cells. In this context it may be mentioned that *A. hydrophila* could grow well with appreciable release of extracellular protease, in chemically defined medium using alanine as the sole source of nitrogen (Pansare et al., unpublished results). It is possible that under conditions of restricted nutrient transport, the synthesis of extracellular protease is first switched off. At higher concentrations of the preservatives, growth is also inhibited. Boethling (1975) observed variable degree of inhibition exerted by chloramphenicol on extracellular protease synthesis in and growth of *Pseudomonas maltophila.* Our findings clearly suggest antimicrobial action of certain food additives not only in terms of growth inhibition but more importantly with respect to attenuation of protease secretion.

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Damage of Amino Acid Residues of Proteins after Reaction with Oxidizing Lipids: Estimation by Proteolytic Enzymes

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ABSTRACT

A mixture of casein and methyl linoleate was stored at 50°C and 80% relative humidity for 0 - 14 days and damage to amino acid residues assessed. The damage was estimated by determining the amino acid composition of the hydrolysate by using proteolytic enzymes (pepsin-pancreatin digestion, followed by aminopeptidase-prolidase hydrolysis). The damage to amino acid residues was the most extensive in methionine, followed by tryptophan, histidine, and lysine. The degree of damage to these amino acids was also determined by chemical methods without using proteolytic enzymes. The efficiency of detecting damage by the present enzymatic method was close to that of the chemical methods.

INTRODUCTION

THE OXIDATION of lipids containing unsaturated fatty acids causes chemical changes in proteins. This chemical reaction between proteins and oxidizing lipids contributes to the deterioration of food proteins during processing, storage and cooking. Such chemical changes in proteins affect not only the nutritional quality of proteins based on the damage of the amino acid residues but also their physical properties owing to aggregation and fragmentation (Cheftel, 1977; Gardner, 1979; Karel, 1980; Funes et al., 1982; Matoba et al., 1982a; Kato et al., 1983).

There have been several publications concerning the damage to amino acid residues. In general, determination of the amino acid composition of proteins has commonly been carried out to assess their nutritional quality, because of simplicity and convenience of the procedures. However, several kinds of chemical methods are required to get accurate results for chemically modified proteins because acid hydrolysis (6N HCl, 110°C), apart from the cleavage of peptide bonds, also causes some undesirable destruction and side reaction of amino acids, thus giving erroneous information (Mauron, 1973; Matoba et al., 1982b). On the other hand, milk enzymatic hydrolysis causes only cleavage of the peptide bonds (Stahmann and Woldegiorgis, 1975; Matoba et al., 1982b).

There have been few studies on amino acid analysis following enzymatic hydrolysis of the protein after reaction with oxidizing lipids (Tannenbaum et al., 1969; Matoba et al., 1982a). In the previous paper (Matoba et al., 1982a), we reported that the damage to amino acid residues of proteins after reaction with oxidizing methyl linoleate was more extensive at relative humidity (RH) 80% than at RH 0%, and methionine, lysine and histidine residues were considerably affected. From our preliminary experiment, tryptophan residue also was susceptible to reaction with oxidizing lipid. In the present paper, we investigated which amino acid residues of these four amino acids is most affected after reaction between casein and oxidizing methyl linoleate at RH 80% and 50°C, using enzymatic hydrolysis (pepsin-pancreatin digestion, followed by aminopeptidase-prolidase hydrolysis). Chemical analyses were also carried out to ascertain the reliability of the results obtianed by the present enzymatic method.

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MATERIALS & METHODS

Materials

Casein (Hammarsten type) was purchased from Merck, Darmstadt. Methyl linoleate was obtained from Tokyo Chemical Industry Co., Ltd., Tokyo. Pepsin and pancreatin were purchased from Sigma Chemical Company, St. Louis. Aminopeptidase (EC. 3.4.11.2) and prolidase (EC. 3.4.13.9), immobilized to Sepharose 4 B, were prepared by the procedure of Matoba et al., (1982b). Florisil was obtained from Nakarai Chemicals Ltd., Kyoto. Methanesulfonic acid (4N) containing 0.2% 3-(2-aminoethyl)indole was purchased from Pierce Chemical Company, Rockford.

Reaction system of casein with methyl linoleate

Methyl linoleate (3g) was added to 3g casein in 150 mL water (pH 7.0). The methyl linoleate was passed through a column of florisil to reduce its initial level of oxidation. The mixture was emulsified in a homogenizer for 3 min at 10,000 rpm. The resulting viscous mixture was quickly frozen in liquid nitrogen, and then freeze-dried. The freeze-dried sample was further dehydrated overnight over P_2O_5 *in vacuo*. The resulting sample was divided into six equal portions. Each portion was kept in a desiccator (2L vol) at a relative humidity of 80%, controlled by saturated $(NH_4)_2SO_4$ solution (Labuza et al., 1976), and stored in an incubator at a constant temperature (50 ± 1°C). After stroage, the mixture was repeatedly extracted with an acetone-methanol mixture (1:1, v/v) by mixing with a mortar and pestle and with a magnetic stirrer to remove the lipid component. The organic phase was removed by centrifugation. The resulting precipitate, which was dried over P_2O_5 *in vacuo* was used for the subsequent experiments. The nitrogen content of the samples was determined with a Kjeldahl nitrogen analyzer (Mitsubishi, KN-01).

Amino acid analysis following enzymatic hydrolysis

Sample (50 mg) was digested in 7.5 mL 0.1N HCl containing 0.75 mg pepsin for 3 hr at 37°C. After neutralization, the mixture was incubated for 20 hr at 37°C with 15 mL 0.1M 3-(N-morpholino)-propanesulfate, pH 8.0, containing 0.025% NaN_3, 2.5 mg pancreatin and 3 mg nor-leucine. A portion (0.5 mL) of the enzymatic digest was further hydrolized in a cartridge containing aminopeptidase-prolidase immobilized to Sepharose 4 B (0.8 mL of the wet gel, containing about 2.5 mg of each enzyme) for 24 hr at 37°C. The buffer used (4 mL) was 0.1M borate, pH 8.0, containing 0.02M $MnCl_2$, 0.025% NaN_3 and 0.03% toluene. The resulting hydrolysate was separated from the immobilized enzymes by suction, and was then subjected to amino acid analysis. An enzyme blank was prepared without the protein samples and incubated under the described conditions. Only trace amounts of amino acids were found in the blank of the immobilized enzymes. The quantitative amino acid determination was carried out with an amino acid analyzer (Hitachi 835 and KLA 5). Nor-leucine was used as the internal standard.

Amino acid analysis by chemical methods

Determination of available lysine. Determination of available lysine was carried out by "the method of difference" according to the procedure of Roach et al. (1967) with some modification as follows: The sample was hydrolyzed by pepsin and pancreatin, as described above, to solubilize it. The hydrolysate (1 mL) was

equally divided into two portions. After lyophilization, one portion was incubated in 2 mL 2% $NaHCO_3$ containing 1% 2,4,6-trinitrobenzenesulfonic acid (TNBS) at 40°C for 2 hr to block amino groups of the hydrolysate, and then refluxed in 6N HCl (4 mL) for 16 hr. The other portion was incubated in the absence of TNBS, then refluxed in 6N HCl for 16 hr. After removal of HCl, free lysine determination of both portions was carried out with an amino acid analyzer described above. An enzyme blank without the protein samples was carried out by incubation under the described conditions. Available lysine content was calculated from the difference between "total lysine conent" (direct HCl hydrolysis) and "residual lysine content" (HCl hydrolysis after blockage by TNBS).

Determination of methionine, tryptophan and histidine. Sample was previously digested by pepsin and pancreatin as described above. The hydrolysate (1 mL), which was lyophilized, was further hydrolyzed with the following appropriate acid or alkali in an evacuated tube. Methionine, tryptophan and histidine were determined using an amino acid analyzer (described above) after hydrolysis with 3.75N NaOH for 16 hr at 110°C (Neumann, 1967), 4N methanesulfonic acid containing 0.2% 3-(2-aminoethyl)indole for 20 hr at 115°C (Simpson et al., 1976) and 6N HCl for 20 hr at 110°C (Moore and Stein, 1963), respectively. An enzyme blank was prepared without the protein samples and incubated under the described conditions. Nor-leucine was used as the internal standard.

RESULTS & DISCUSSION

A MIXTURE of casein and methyl linoleate was stored at 50°C and 80% RH for 0–14 days and damage to methionine, lysine, tryptophan and histidine residues of the reacted casein was assessed by amino acid analysis following enzymatic hydrolysis (enzymatic method) and chemical methods (Table 1). The evaluation of damge to methionine, lysine and tryptophan residues by the enzymatic method very close to that obtained by the chemical methods. On the other hand, the concentration of histidine measured by the enzymatic method was lower than that by the chemical method. However, a similar tendency to damage, following reaction with methyl linoleate, was indicated by both methods. At present it is not clear why this difference in histidine detection was observed.

The protein reacted with oxidizing lipid at 80% RH was scarcely soluble in aqueous solution. Therefore, the hydrolysate of the reacted protein following pepsin and pancreatin hydrolysis was used as the sample for determining the available lysine content. We used 2,4,6-trinitrobenzenesulfonic acid (TNBS) to block amino groups but not 2,4-dinitrofluoro benzene (DNFB) (procedure of Roach et al.), because TNBS has better solbulity than DNFB in the reaction mixture, Holsinger and Posati (1975) indicated that TNBS was used as a blocking reagent of amino groups, but ϵ-TNP lysine did not separate from free lysine during the chromatographic step. However, our chromatographic step with the amino acid analyzer gave a good separation of free lysine from TNBS derivatives. Under the present conditions, free lysine was allowed to react with TNBS and the modified lysine was then hydrolyzed with 6N HCl, resulting in release of 5% free lysine from the TNP-lysine. We applied Neumann's method (1967) for determination of methionine of the reacted casein, because methionine sulfoxide has been known to revert largely to methionine by 6N HCl hydrolysis (Keutmann and Potts, 1969). After alkaline hydrolysis (3.75N NaOH, 110°C hr) of free methionine and methionine sulfoxide, the recovery was 100% and 80%, respectively. Methionine sulfoxide did not revert to methionine during this alkaline hydrolysis. Therefore, the determination of methionine by this method would give accurate information regarding the loss of methionine residue from the reacted casein.

Fig. 1 summarizes each amino acid loss measured by the enzymatic method. The damage to amino acid residues of the reacted casein was most extensive to methionine, followed by tryptophan, histidine and lysine. Gardner (1979) reported in his review that many kinds of amino acid residues were damaged after the reaction of proteins with oxidizing lipids under drastic conditions. However, we observed browning and strong off-odor after 10 days of the reaction. Such a color and off-odor would not be sensorily acceptable qualities for foodstuffs. Amino acid residues otehr than methionine, tryptophan, lysine and histidine were not considerably effected after 8 days of the reaction. Therefore, the above four amino acid residues may be damaged after the exposure of proteins to oxidizing lipid under actual conditions for foodstuffs, and methionine is the most susceptible. Methionine and methionine sulfoxide were detected with an amino acid analyzer in the hydrolysate of the reacted casein by the alkaline or enzymatic hydrolysis, and trace amounts of emthionine sulfoxide and methionine sulfone other than methionine were detected following acid hydrolysis (6N HCl). The methionine content obtained by 6N HCl hydrolysis was similar among all the samples. Therefore, it is clear that methionine was predominantly converted to methionine sulfoxide, as suggested by Tannenbaum et al. (1969).

As Stahmann and Woldegiorgis (1975) stated, it would seem that a measure of the amount of amino acid liberation from proteins by in vitro proteolytic enzymes could provide a good index of their qualities, since high quality proteins are those that are well digested in vivo. The present in vitro enzymatic system was a two step system composed of pepsin-pancreatin digestion which was a model of the hydrolytic system before intestinal absorption, whereas aminopeptidase-prolidase hydrolysis was that of the intestinal mucosa (membrane digestion) and after intestinal absorption (intracellular hydrolysis). All the proteolytic enzymes used in the experiment are available commercially. The immobilized proteases were very stable, and did not lose their activities even after being used a hundred times over a period of one year or more. Therefore, the present hy-

Table 1—Comparison of enzymatic and chemical methods for the determination of amino acid residues of casein after reaction with oxidizing methyl linoleate[a]

		mmoles/16g N					
		day					
		0	4	6	8	10	14
Methionine	Enz.[b]	20.7 (100)[c]	19.0 (92)	13.4 (65)	8.8 (43)	1.0 (5)	0 (0)
	Chem.[b]	20.5 (100)	19.1 (93)	14.2 (69)	9.2 (45)	0.2 (1)	0 (0)
Lysine	Enz.	63.2 (100)	62.4 (99)	57.8 (91)	57.4 (91)	40.9 (65)	25.2 (40)
	Chem.	62.0 (100)	61.0 (98)	61.1 (98)	52.7 (85)	42.9 (69)	29.7 (48)
Tryptophan	Enz.	6.3 (100)	6.3 (100)	4.5 (71)	3.4 (54)	3.0 (48)	2.5 (40)
	Chem.	6.4 (100)	6.2 (97)	5.2 (81)	4.9 (76)	3.5 (55)	3.5 (55)
Histidine	Enz.	14.5 (100)	14.0 (97)	14.5 (100)	15.1 (104)	8.1 (56)	5.0 (34)
	Chem.	20.7 (100)	20.9 (101)	19.3 (93)	18.5 (89)	12.9 (62)	10.3 (50)

[a] A mixture of casein and methyl linoleate was stored at 50°C and RH 80% for 0 - 14 days.
[b] Enz., enzymatic method; Chem., chemical methods.
[c] ( ), remaining amino acid residue (%).

drolytic system could be a useful tool for the simple and convenient nutritional evaluation of foodstuffs, especially food proteins modified chemically during their processing, storage and cooking.

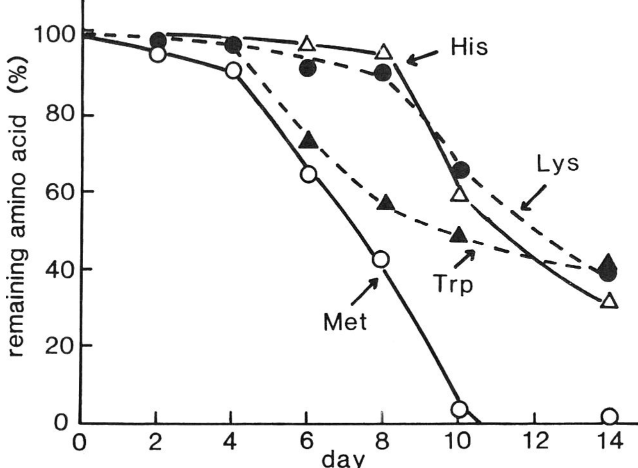

Fig. 1—Damage to amino acid residues in casein after reaction with oxidizing methyl linoleate at 50°C and 80% RH. [Remaining amino acid residue (%), see Table 1.]

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Pectolytic Enzymes in Sweet Bell Peppers (*Capsicum annuum* L.)

JOSEPH J. JEN and MICHELE L. ROBINSON

ABSTRACT

Low levels of polygalacturonase (PG) and pectinesterase (PE) activities were quantitated in sweet bell peppers at four stages of maturity based on surface ground color (dark green, light green, turning, and red). The PG activity increased during pepper ripening and was maximal at the turning stage while the PE activity declined during ripening and was maximal at the light green stage. Pepper texture measurements were found to decline concomitantly with the increase in PG activity. Pepper PG showed pH optimum between 4.8 to 5.0 and increased temperature stability with advanced ripening. Compared to tomatoes, pepper PG activity was 116- to 164-fold lower and pepper PE activity was 429- to 1900-fold lower than the corresponding tomato enzymes.

INTRODUCTION

SWEET PEPPER FRUITS (*Capsicum annuum* L.) are normally sold in markets either as green or as red bell peppers. The texture, in particular the crispness of peppers, is an important quality attribute to consumers. It is known that vegetable texture is closely related to the pectic substances and to activities of pectolytic enzymes (Doesberg, 1965).

The role of pectolytic enzymes in tomato texture and ripening has been well studied. Tomato softening is accompanied by increased activities in pectinesterase (PE; Hobson, 1963) and polygalacturonase (PG; Hobson, 1964; Grierson et al., 1981). Hobson (1965) showed that softening of tomatoes occurs concomitantly with advanced ripening. In addition, the loss of pectic substances from tomato cell walls during softening has been correlated with the action of PG (Sawamura et al., 1978). Tomato PG is absent in green tomatoes and appears to be synthesized during ripening with activity first appearing at the onset of coloration (Hobson, 1963; Grierson et al., 1981). Tucker and Grierson (1982) reported that the increase in PG activity during tomato ripening is due to *de novo* protein synthesis. Tigchelaar et al. (1978) have proposed that PG activity may initiate ripening. The PE activity may be involved in the softening phenomenon by influencing PG activity. Tomato PE activity was shown to be present in green fruit and increased during ripening, reaching maximal levels at the ripe fruit stage and declined thereafter (Grierson et al., 1981).

There is little information in the literature on the pectolytic enzymes of bell peppers. One reason such information is lacking is probably due to the anticipated low enzyme activities in peppers, as the texture degradation in peppers is a slow process. Gross (1982) recently described a highly sensitive assay method for PG activity in tomatoes. We report here an adaptation of Gross' method to study this enzyme in fresh bell peppers during the ripening process. We also report the PE activities in peppers and the relationship between pectolytic enzyme activity and bell pepper texture. Comparison of pepper and tomato PG activities and properties and PE activities were also investigated.

MATERIALS & METHODS

Fruits

During the summers of 1982 and 1983, peppers were freshly harvested from a local farm, transported to the laboratory, and divided into four-groups based on surface ground color (dark green, light green, turning, and red). For each experiment, peppers from each group were diced into 0.5 in. pieces. A 100g sample from each group was randomly chosen and extracted and assayed for PG and PE activities. Six 50g samples were randomly chosen for Instron texture analysis.

Enzyme extractions

Preparation of enzyme extracts was similar to the methods of Pressey and Avants (1972) for tomato and Paynter and Jen (1974) for peach. A 100g diced pepper sample from each group was homogenized in 100 mL distilled deionized water for 1 min in a Waring Blendor. The resulting homogenate was filtered through two layers of cheesecloth. The residue was resuspended in 100 mL 1M NaCl and further broken up by Polytron (Brinkmann) for 1 min. The resulting suspension was adjusted to pH 6.0 using 1N NaOH and incubated with stirring at 4°C for 1 hr. The suspension was filtered through two layers of cheesecloth. The filtrate was centrifuged at 27,000 × *g* for 15 min at 4°C. The resulting supernatant was desalted on a Spehadex G-25 column and used for the assay of PG and PE activities. Freshly harvested tomatoes at turning and red stages of maturity were extracted and assayed by the same procedure.

Enzyme assays

Tomato and pepper PG activities were measured by the method of Gross (1982) which uses 2-cyanoacetamide to measure nanomolar quantities of reducing sugar formed. For pepper PG activity, 22 hr of incubation at 40°C was used instead of 1 hr for tomato PG. The resulting reaction product was measured at OD_{276} using a Beckman DU-8 recording spectrophotometer in the kinetics mode. The blank contained all solutions except that the addition of the enzyme extract was after the addition of the pH 9.0 borate buffer. One unit of PG activitiy was defined as the amount of enzyme capable of catalyzing the formation of 1 nanomole of reducing sugar per minute under the assay conditions.

The PE activity was measured using a modification of the method of Rouse and Atkins (1955). The rate of citrus pectin demethylation was measured at room temperature by titration with 0.025N NaOH using a Brinkmann automatic titration unit. Fifty mL 1% pectin in 0.1N NaCl was used as substrate and adjusted to pH 7.0 before addition of 1–5 mL pepper enzyme extract or 50 μL tomato extract. One unit of PE activity was defined as the amount of enzyme capable of catalyzing the consumption of 1 micromole of base per 10 min under the assay conditions.

Texture measurement

Texture measurements were conducted using an Instron model 1132 texture analyzer. Shear force was measured using a 5000 kg ring and a stroke speed of 20 cm/min. Computer analysis of data showed that the maximum peak height was a better firmness index than the peak area for bell peppers. The firmness was expressed as kg shear force per g pepper.

RESULTS & DISCUSSION

PEPPER pectolytic enzyme activities (PG and PE) were measured at four stages of ripeness. As shown in Fig. 1, pepper PE activity was present at the dark green stage and was maximal at the light green stage. The PE activity declined with further ripening and was negligible at the turning and red stages. The pepper PG activity developed during ripening and was maximal at the turning and red stages. From these results, it appears that the action of PE is to prepare the pepper pectic substances for PG to act upon. Although not conclusively known, the degree of pectin methylation appears important for the action of PG (Doesburg, 1965). The pepper pectolytic enzyme activities in ripening peppers par-

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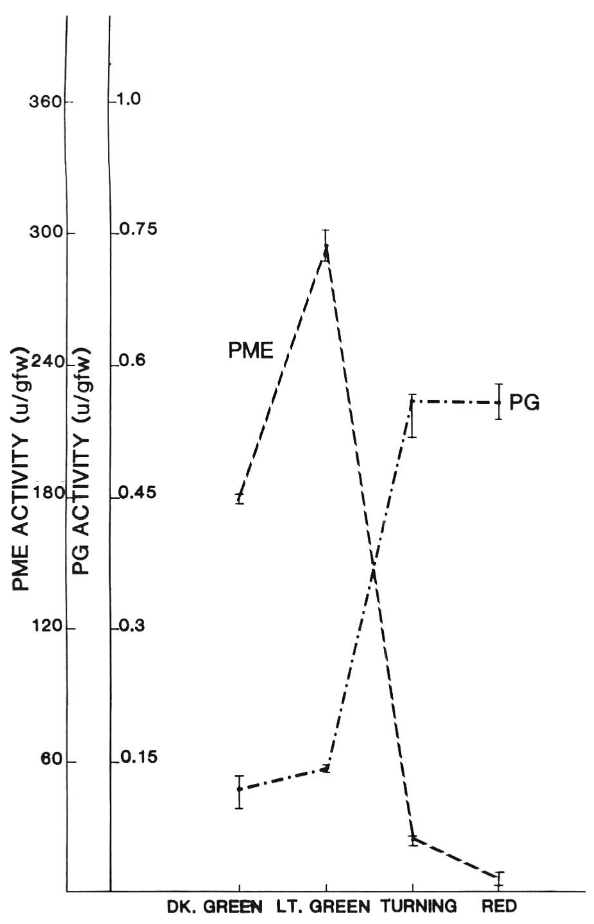

Fig. 1—Pepper pectolytic enzyme activity during ripening. PG = polygalacturonase; PME = pectinesterase. Vertical bars indicate the range of enzyme activities.

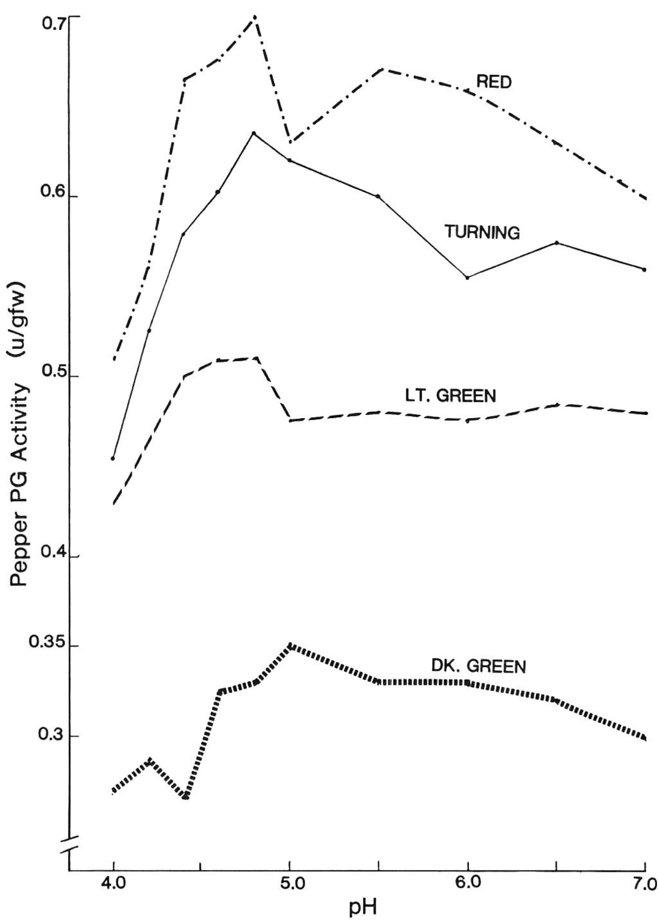

Fig. 2—pH curves of pepper PG at various stages of ripening.

Table 1—Comparison of pepper and tomato pectolytic enzymes

Stage of ripeness	PE activity (Unit/g)	Fold difference	Activity (Unit/g)	Fold difference
Turning tomato	9,585.05		64.86	
		429		116
Turning pepper	22.35		0.56	
Red tomato	15,737.70		91.91	
		1900		164
Red pepper	8.29		0.56	

Table 2—Texture analysis of ripening peppers

State of ripening	PE activity (Unit/g)	PG activity (Unit/g)	Shear force (kg/g)	Texture loss (%)
Dark green	174.45	0.117	47.5	0
Light green	295.49	0.143	45.8	3.6
Turning	22.35	0.561	41.6	12.6
Red	8.29	0.559	40.0	15.8

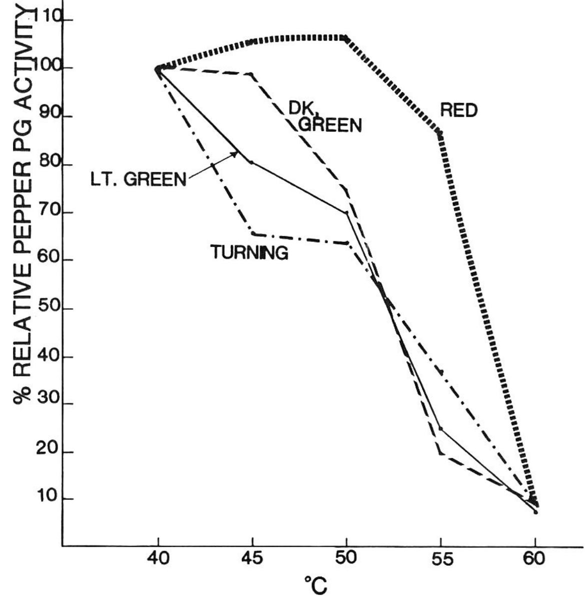

Fig. 3—Thermal stability of pepper PG at various stages of ripening.

alleled those of tomato pectolytic enzymes (Grierson et al., 1981). The decline in pepper PE activity may be used to regulate pepper PG activity by limiting the affinity between pepper PG and the pectic substances.

Pectolytic enzyme activity, particularly PG activity, was quite low in the bell peppers. It was only possible to measure the low level of PG activity in the peppers due to the use of Gross' method (1982). This method is capable of measuring as little as 1 nanomole of reducing sugar formed. A problem encountered with the

Gross method was the presence of interfering compounds in the pepper extracts which gave high absorbance readings at 276 nm. Interference made it difficult to differentiate between specific and nonspecific absorption of 276 nm. The problem was alleviated by including enzyme extract in the reaction blanks. The Gross method for reducing sugar analysis is superior to the commonly used Nelson method. The Gross method is about 1000 times more sensitive and is easier to execute than the Nelson method when large numbers of samples are involved.

Peppers were compared with tomatoes because they both belong to the *Solanaceae* family. Table 1 shows the pectolytic enzyme activities (PG and PE) at the turning and red stages for peppers and tomatoes. Tomato enzyme activities were much higher at both stages of ripening than the pepper enzymes at the corresponding stages. Turning tomato PE activity was 429-fold higher than turning pepper PE and red tomato PE was 1900-fold higher than red pepper PE. Tomato PG activity was higher than pepper PG activity; however, the magnitude of difference was not as great as that for PE. Turning tomato was 116-fold higher than turning pepper PG and red tomato was 164-fold higher than red pepper PG. The large difference in pectolytic enzyme activities may be one reason why the two vegetables differ significantly in texture and softening rate in the ripening process.

Pepper texture was measured by Instron analysis as shown in Table 2. The pepper texture index declined with advanced ripening, however, the overall texture loss of 15.8% was not great. The loss in pepper texture was found to be consistent with the increase in pepper PG activity. A 3.9-fold increase in pepper PG activity corresponded to a 3.4-fold loss in texture. Softening in the pepper does not correlate with PE activity, as PE activity declined as softening increased. The decline in tomato texture during ripening has also been correlated with increase in PG activity (Sawamura et al., 1978). Consistent with our results for pepper, the tomato softening does not correlate with PE activity, as Nr mutants soften slowly but have normal PE levels (Tucker et al., 1982).

At all stages of ripening, pepper PG had a pH optimum between pH 4.8 and 5.0 (Fig. 2). In addition, at the red stage of ripeness, pepper PG appears to have a second pH optimum between pH 5.5 and 6.0. The double pH optima of red pepper may be indicative of the presence of two isoenzyme forms. The development of PG isoenzyme forms during tomato ripening has been well established (Tucker et al., 1980). Tomato PG has a more acidic pH optimum of pH 4.4 at the turning and red stages and is more pH sensitive than the pepper enzyme at the corresponding stages of ripeness (Author's unpublished data).

Pepper PG at the dark green, light green, and turning stages was less stable to temperature above 40° than red pepper PG (Fig. 3). The red pepper PG was activated by temperatures of 45°C and 50°C. The increased thermal stability of red pepper PG may be indicative of the presence of more than one isoenzyme form. The two tomato PG isoenzyme forms present in the fully ripe fruit have very different thermal stabilities (Pressey and Avants, 1973). Regardless of ripeness, pepper PG was nearly completely inactivated at 60°C in 20 minutes. Red tomato PG also showed activation by temperatures greater than 40°C. Tucker et al. (1981) have shown that the PG isoenzyme, PG-2, present in ripe tomatoes was more temperature stable than the PG isoenzyme, PG-1, present in the turning tomato.

In conclusion, pectolytic enzymes do exist in sweet bell peppers but at low levels. The pepper enzymes have properties and characteristics similar to the tomato pectolytic enzymes.

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Effect of Sweetener Type and Lecithin on Hygroscopicity and Mold Growth in Dark Chocolate

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ABSTRACT

Storage changes in dark chocolate samples sweetened with five different carbohydrate sweeteners, namely, sucrose β-D-fructose, maltose hydrate, L-sorbose and sorbitol at two added levels of the surfactant lecithin were evaluated by hygroscopicity measurements at different relative humidities (RH) and for mold damage at 100% RH. Sorption isotherms of the samples showed that β-D-fructose and sorbitol are particularly unsuitable in themselves as chocolate sweeteners because of their wide humectant ranges while only L-sorbose compared adequately with sucrose and showed no mold damage after 10 days at 100% RH. The importance of the surfactant lecithin as an additive particularly in chocolate products designed for high humidity conditions e.g. in the tropics, is also discussed.

INTRODUCTION

DURING SHELF-LIFE, either in storage or in the retail channel, chocolates may absorb moisture under extremes of atmospheric humidity, particularly in highly humid tropical conditions. This property of moisture absorption under normal atmospheric conditions, known as 'hygroscopicity', is characteristic of any dry but water-soluble crystalline solid.

Different carbohydrate sweeteners are known to absorb water at different rates (Grover, 1949; Mahdi and Hoover, 1965) although the extent to which carbohydrates might influence the humectant behavior of chocolate has not been widely investigated.

Many different carbohydrate sweeteners and sweetener mixtures are now finding increasing use in the chocolate industry both for different designs as well as sucrose replacement. However, sugar bloom which causes chocolate to have a grey surface appearance is a frequent defect in storage under conditions of high humidity (⩾80% RH).

Thus investigations of humectancy of carbohydrate sweeteners in food products will give very valuable information especially since the various interaction effects during food processing are known to cause physicochemical modifications (Høyem and Kvale, 1977).

Harris (1968) suggested that since surfactants used in chocolate act mainly at the sugar surface, they might enhance the humectancy of chocolate products, thus enabling them to withstand higher humidities without damage. Easton et al. (1952) had shown that certain sorbitan and polyoxyethylene sorbitan esters of fatty acids, when present in chocolate up to 1% can cause significant retardation of certain types of fat bloom. These additives have also been shown to control crystallization in confectioners' coatings and thus confer properties of better gloss and longer shelf-life (Drew, 1962; Ziemba, 1966).

The objective of this study was to investigate the hygroscopic properties of some food carbohydrate sweeteners and sweetener-surfactant interaction effects on common storage problems.

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MATERIALS & METHODS

ALL SAMPLES of carbohydrate sweeteners used in this study were of analytical grade. These were: sucrose (obtained from May & Baker Ltd., Dagenham, England), maltose hydrate, β-D-fructose, L-sorbose and sorbitol (obtained from Sigma Chemical Co., England). The salts (magnesium chloride hexahydrate, hydrated potassium carbonate, sodium chloride, and ammonium sulphate) were also obtained from Sigma Chemical Co., England.

Preparation of chocolate samples

The dark chocolate samples used in this study were prepared as described by Ogunmoyela and Birch (1984) based on the same ingredient composition of sweetener = 50.0%, cocoa liquor = 45.0%, butter oil = 4.0%, cocoa butter = 0.5% and days lecithin = 0.5%, but with the cocoa butter replaced by 0.5% lecithin in a second set of samples which contained 1.0% soya lecithin. Final moisture content and equilibrium relative humidity were determined on the prepared samples according to Pearson (1976).

Samples to be used for storage tests were subsequently ground in a mortar and spread out thinly in aluminium dishes under phosphorus pentoxide in a desiccator for drying.

Samples of commercial "Bournville" dark chocolate were also prepared in a similar manner for comparison with the test samples.

Hygroscopicity measurements

A procedure based on the method described by Minifie (1980) was used.

One gram of each of the dried samples was accurately weighed onto shallow aluminium dishes and placed in four Townson and Mercer Humidity cabinets maintained at 33, 45, 75 and 80% RH, respectively, in a constant temperature room. The atmospheres were achieved by placing saturated solutions plus excess solutes of magnesium chloride hexahydrate, hydrated potassium carbonate, sodium chloride and ammonium sulphate respectively in each of the four cabinets at 24.5°C for 96 hr to reach equilibrium, before introducing the samples into the cabinets.

Samples were carefully removed for weighing every 48 hr over a 14-day period by which time constant weights had usually been attained.

The measurements were carried out separately for samples containing 0.5 or 1.0% lecithin, respectively, in duplicate and replicated twice. Moisture uptake or moisture gain of the samples were calculated from the expression (gain in weight of sample/dry weight of sample × 100%).

Mean moisture uptake was calculated from the average of the two replicate measurements. Mean moisture uptake at equilibrium obtained from the plots of percent moisture absorption against period (days) at different atmospheres were used for plotting the sorption isotherms of the different chocolate samples.

Dark chocolate samples ground and dried were also kept in a saturated atmosphere at 100% RH. The samples were then examined for visible mycelial growth which occurred after 10 days.

RESULTS & DISCUSSION

MOISTURE GAIN or loss during storage accounts for quality changes in many confectionery products which may be desirable or undesirable (Mahdi and Hoover, 1965). Obviously in a sugar-fat mixture as in chocolate where interactions between the sugars and surfactants particularly, may govern some of the physical effects observed, the moisture uptakes of the carbohydrate sweeteners will be affected.

Fig. 1 shows the plots of percent moisture absorbed against time at the different RH. Sorbitol and β-D-fructose

samples appear to be particularly hygroscopic especially at the higher humidities, and less obviously, maltose hydrate. Table 1 shows the final moisture contents and equilibrium relative humidities of dark chocolate samples. Only the values for β-D-fructose are strikingly different whereas sorbitol and maltose hydrate are also shown in Fig. 1 to be hygroscopic. However, L-sorbose samples compare most favorably with sucrose samples in their final moisture content and uptake.

Generally, the value of any nutritive sweetener as a humectant can be estimated not only from its rate of moisture gain or loss when subjected to a change of environment or its equilibrium moisture content at different RH, but also the range of equilibrium moisture contents over a range of RH, that is, its sorption isotherm. Humectants having a wide range would be very hygroscopic in moist surroundings but would dry out rapidly in drier conditions, although the rate of moisture gain may be different from the rate of moisture loss. Thus, the desirability of any humectant in products like chocolate would depend on its ability to confer on the product, a narrow humectant range. When the equilibrium moisture contents at different RH were plotted as in Fig. 2, the wide humectant ranges of β-D-fructose and sorbitol samples became very obvious. However, the level of addition of the surfactant lecithin did not appear to affect this humectant behavior as there were no appreciable differences between the two levels of addition in terms of the isotherms obtained.

In assessing the probable role of the higher concentration of surfactant in enabling chocolate to withstand higher humidities without damage, since these additives exhibit strong surface effects, it would appear that they could act as a surface coating to the sugar, restricting its availability to microbial attack in storage. Thus, samples kept at 100% RH were examined for mold growth daily inside the cabinet without exposure and the experiment was discontinued at the first visible occurrence of mold growth, which occurred after 10 days. When the mold growth on 1g dark chocolate samples containing either 0.5% or 1.0% lecithin was compared, the samples containing the higher concentration of

—Continued on page 1142

Table 1—Final moisture contents and equilibrium relative humidities of sample dark chocolates

Sweetener	Final moisture content (%)	ERH (%)[a]
Sucrose	0.85	32
Maltose hydrate	3.83	33
β-D-fructose	6.03	45
L-sorbose	1.21	34
Sorbitol	1.05	35

[a] Equilibrium relative humidity

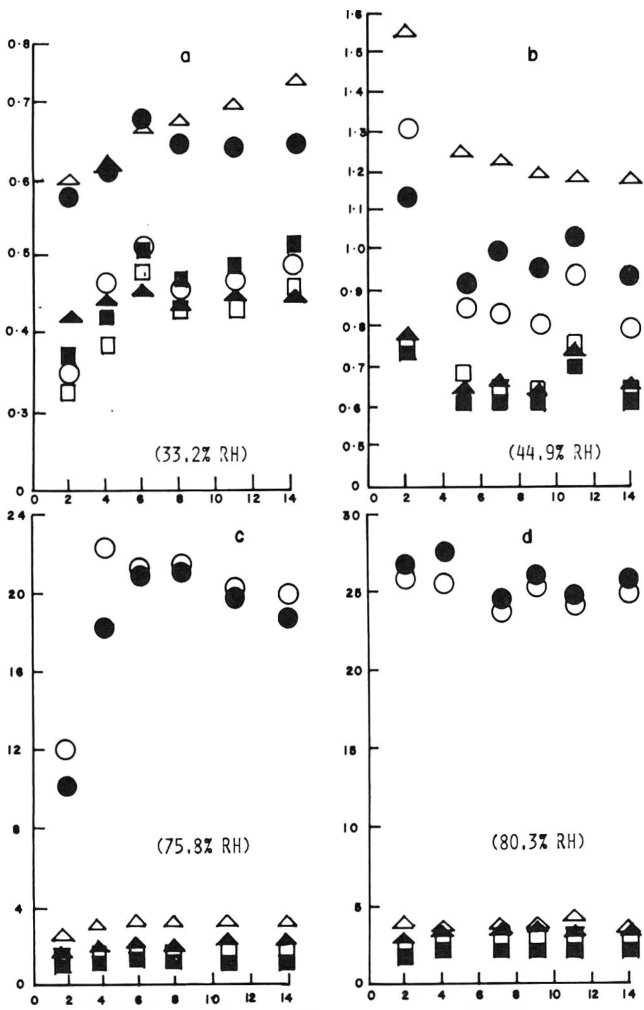

Fig. 1—Plots of percent moisture absorbed versus time (days) at different relative humidities. The data are for dark chocolate samples containing 0.5% lecithin. ○ = β-D-fructose; ● = sorbitol; △ = maltose hydrate; ▲ = L-sorbose; □ = sucrose, and ■ = Bournville chocolate.

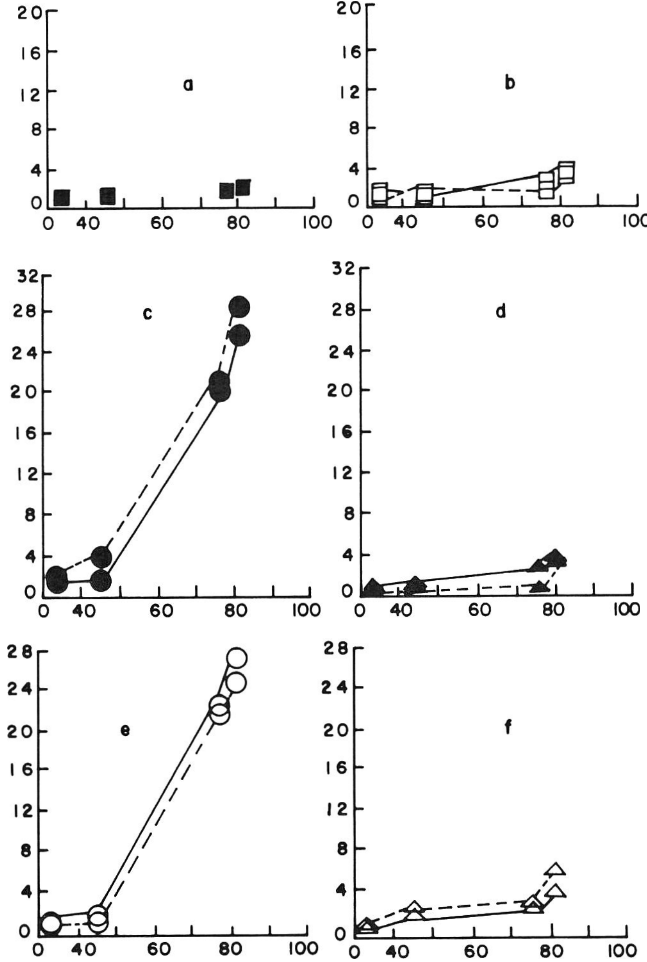

Fig. 2— Plots of equilibrium moisture content (%) versus percent relative humidity for chocolates containing different carbohydrate sweeteners and two lecithin concentrations. (a) Bournville chocolate; (b) sucrose; (c) β-D-fructose; (d) sorbitol; (e) L-sorbose; and (f) maltose hydrate. (——) represents 0.5% added lecithin; (- - - -) represents 1.0% added lecithin.

(-)-Epicatechin Content in Fermented and Unfermented Cocoa Beans

H. KIM and P. G. KEENEY

ABSTRACT

As determined by high performance liquid chromatography, (-)-epicatechin concentrations among freshly harvested beans of verified genetic origin ranged from 21.89–43.27 mg/g of dry defatted samples. Fermented beans showed much lower concentrations (2–10 mg). During fermentation, a trend towards decrease in (-)-epicatechin content was observed. Commercial beans from areas with reputations for shipping well-fermented products contained lower levels of (-)-epicatechin than beans from regions where fermentation is less extensive.

INTRODUCTION

COCOA BEAN POLYPHENOLS, comprising 12–18% of whole dry bean weight, have long been associated with the flavor and color of chocolate. Through complex chemical interactions of polyphenols with proteins (tanning), astringency is reduced and the burnt feather character of roasted protein is depressed (Bracco et al., 1969; Quesnel, 1966; Roelofsen, 1958). Furthermore, oxidation of polyphenols results in the formation of melanin pigments.

Approximately 35% of the polyphenol content of unfermented Forastero cocoa beans is (-)-epicatechin (3,3',4',5,7-pentahydroxyflavan) (Forsyth, 1955; Forsyth and Quesnel, 1963). During fermentation and drying, this compound undergoes major changes. Subsequent to bean death, usually 24–48 hr into fermentation, (-)-epicatechin diffuses from its storage cells and undergoes oxidation and polymerization to form complex tannins (Forsyth and Quesnel, 1963; Roelofsen, 1958). During the drying stage, enzymatic or spontaneous oxidation of (-)-epicatechin leads to the formation of melanin and melanoproteins. This results in the characteristic browning of fermented cocoa beans.

Utilizing a high performance liquid chromatography (HPLC) method (Kim and Keeney, 1983), an investigation was conducted to determine (-)-epicatechin concentrations among several varieties of cocoa beans. Also, the effects of fermentation on (-)-epicatechin content were studied.

MATERIALS & METHODS

Source of samples

Fermented and unfermented Trinidad-Jamaican hybrids, Catongo, and Forastero cocoa beans were provided by Paulo Berbert, Centro de Pesquisas de Cacao (CEPEC), Itabuna, Bahia, Brazil. Samples were also available for analysis from the Penn State collection of commercial shipments of cocoa beans from major producing regions. A fermentation series of Trinidad-Jamaican hybrids was supplied by Stanley M. Tarka, Hershey Foods Corporation, Hershey, PA. The fermentation series (a cross between IMC-67 Catongo and SIC-831 Forastero) used by Timbie et al. (1978) was analyzed, as were freeze-dehydrated beans from pods of authenticated genetic clones supplied by J. Soria, CATIE, Turrialba, Costa Rica.

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Determination of (-)-epicatechin

Shell-free dry beans were ground in an analytical mill and defatted with hexane for 16-18 hr. For the extraction of (-)-epicatechin, 0.5g defatted sample was sonicated with 80 mL aqueous acetone. The resulting extract was filtered and the residue and all glassware were washed with 80% aqueous acetone to bring the volume of extract to 100 mL. A portion of this extract (5 or 10 mL) was dried on a rotary evaporator and the residue, after resuspending in 10 mL distilled water, was injected through a reverse-phase C_{18} SEP-PAK cartridge (Waters Associates, Milford, MA). (-)-Epicatechin retained in the cartridge was eluted with 10 mL 40% aqueous methanol, and 10 μL of this solution were injected into the liquid chromatograph. For analysis of cocoa shells, samples were ground in a mortar and pestle prior to extraction.

The liquid chromatograph included a Waters Associates model 6000-A pump, U6K Universal injector, and Model 450 variable wavelength detector. Separation of (-)-epicatechin was accomplished on a μBondapak C_{18} reverse-phase analytical column with a mobile phase of water:methanol:acetic acid (87:8:5) pumped at a flow rate of 2.0 mL/min. The eluting epicatechin was detected by monitoring absorbance at 280 nm and quantified by comparing peak height of the sample to those obtained from authentic standards.

RESULTS & DISCUSSION

(-)-Epicatechin concentration in unfermented cocoa beans

Data for (-)-epicatechin in Table 1 are for 8 clones representing 4 varieties of cocoa beans. Except for Trinidad-Jamaican hybrid beans, the samples had been freeze-dried immediately after removal from the pods. Thus, they represent freshly harvested beans unaffected by any post-harvest treatments. The SIC-250 clone of Amazon Forastero had the highest concentration of (-)-epicatechin while ICS-Type had the lowest. Although statistical analysis (Tukey's ω method of mean separation at 95% confidence level) showed significant differences among the freeze-dried samples, the extent of these differences was, nevertheless, of questionable practical importance.

For the Trinidad Jamaican hybrid in Table 1, (-)-epicatechin content was only about 50% that of the other samples. This could be credited to a variance caused by post-harvest handling rather than bean type. Whereas immediate freeze-

Table 1—Concentration of (-)-epicatechin in cocoa beans unaffected by post-harvest variables[a]

Clone	Varietal type	(-)-Epicatechin conc[b] in defatted sample (mg/g)
NA-22-23	Nacional	37.63 ± 0.22^{g}
N-22-A3	Nacional	34.65 ± 0.20^{ij}
UP-667	Trinitario	41.33 ± 0.54^{de}
UF-11	Trinitario	39.33 ± 0.71^{ef}
UF-296	Trinitario	36.33 ± 1.30^{gh}
EEG-29	Amazon Forastero	35.33 ± 1.18^{hij}
SIC-250	Amazon Forastero	43.27 ± 0.44^{d}
ICS-TYPE	Trinidad-Jamaican Hybrid	21.89 ± 0.26^{k}

[a] Pods opened, testa removed, and cotyledons freeze dehydrated.
[b] Mean of three different sample preparations (duplicate injections of each preparation) ± standard deviation. Values followed by a common letter are not significantly different ($P \geq 0.05$) — Tukey's ω method of mean separation.

drying after removal from the pods characterized the other bean types, the Trinidad-Jamaican hybrid had been heated to an internal temperature of 105°C for 5 min with microwave energy prior to sun-drying for 2 days. The intent of the microwave treatment, conducted in another investigation by Tarka (1980), was rapid inactivation of polyphenol oxidase (PPO). Griffith (1957) noted that (-)-epicatechin was the major substrate of PPO for oxidative browning reactions during the drying stage. Evidently, PPO in the Trinidad-Jamaican hybrid was not effectively inactivated by the microwave treatment, and during 2 days of sun-drying oxidation of (-)-epicatechin, mediated by PPO, caused a reduction in (-)-epicatechin content.

Table 2—Concentration of (-)-epicatechin in cocoa beans from shipments representing several countries of production

Bean source	(-)-Epicatechin conc in defatted sample[a] (mg/g)
Ivory Coast	6.22
Maracaibo (Venezuela)	3.62
Samoa	10.64
Trinidad	4.68
Bahia (Brazil)	8.23
Ghana	4.52
Lagos (Nigeria)	4.68
Costa Rica	16.52
Arriba (Ecuador)	8.08
Jamaica	2.66

[a] Mean of duplicate injections of a single extract.

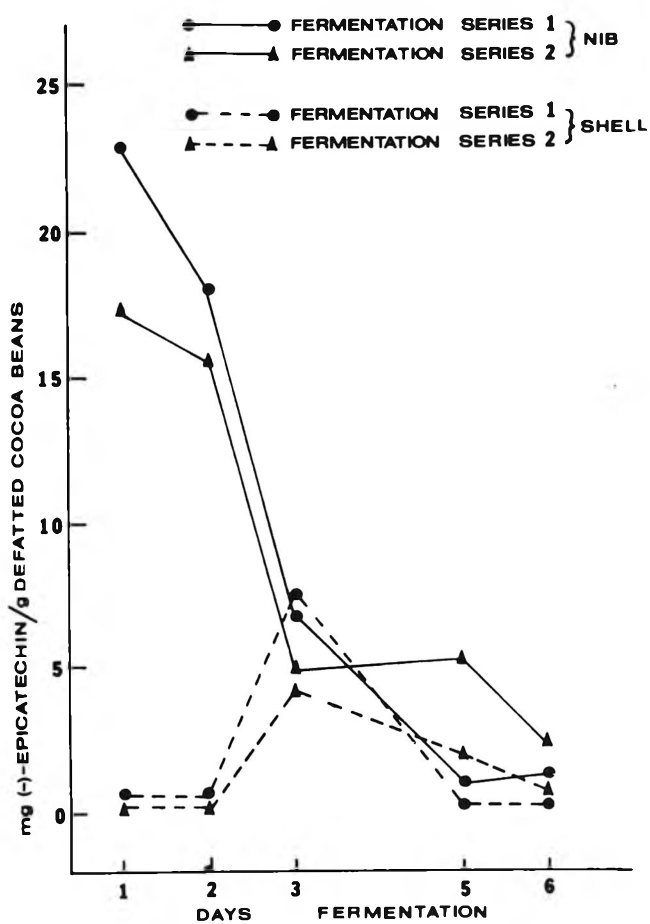

Fig. 1—Changes in (-)-epicatechin of Trinidad-Jamaican hybrid cocoa beans during fermentation.

(-)-Epicatechin in cocoa beans from different regions of production

(-)-Epicatechin data obtained through analyses of commercial samples of cocoa beans are listed in Table 2. Although most beans for chocolate manufacture are fermented, it is far from being a standardized process throughout the world, or even within a region, as evidenced by a 6-fold variation in (-)-epicatechin concentration among the samples.

Caution should be exercised in interpreting the data in Table 2 since only a single lot from each country was sampled in the survey. Interestingly, however, beans from regions with reputations for fully fermented products, such as African countries and Trinidad, had lower concentrations of (-)-epicatechin than Costa Rican and Ecuadorian beans which usually fall into the lightly fermented category.

Fermentation studies

Concentration changes for (-)-epicatechin in nibs and shells of Trinidad-Jamaican hybrid cocoa beans during the fermentation trials in Belize (Hershey Foods Corp. Experimental Station) are illustrated in Fig. 1. Loss of (-)-epicatechin in the cotyledon (nib) concur with the findings by Forsyth (1952). The sharp decrease between the second and third day is of interest. According to Roelofsen (1958), diffusion of polyphenols from their storage cells begin upon bean death, which usually occurs at about this time.

Diffusion of (-)-epicatechin leads to complex chemical changes within the cotyledon, most notably, polymerization of (-)-epicatechin to form complex tannins. However, migration of (-)-epicatechin into the shell may also take place (Roelofsen, 1958; Forsyth and Quesnel, 1963). This is suggested by the observed increase in (-)-epicatechin within the shells between the second and third day. Probably, both polymerization and loss into fermentation sweatings account for the continuing decline in (-)-epicatechin concentrations in nib and shell during the latter stages of fermentation.

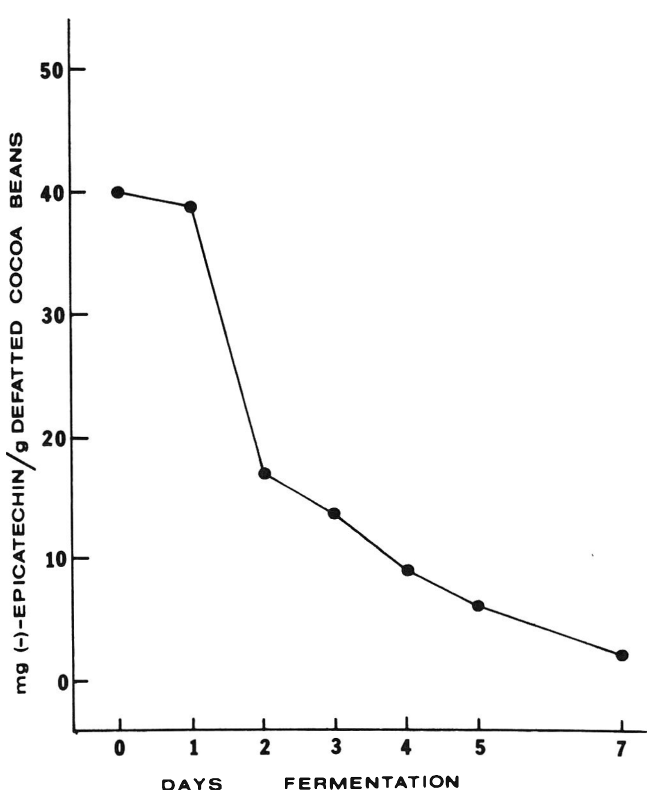

Fig. 2—Changes in (-)-epicatechin of Trinidad cocoa beans during fermentation.

Table 3—Concentration of (-)-epicatechin in fermented cocoa beans from experimental clones

Clone	Varietal type	(-)-Epicatechin conc in defatted sample[a] (mg/g)
ICS-1	Trinidad-Jamaican Hybrid	10.03 ± 0.20[c]
ICS-G	Trinidad-Jamaican Hybrid	3.42 ± 0.05[d]
SIC-325	Amazon Forastero	2.32 ± 0.19[e]
SIC-864	Amazon Forastero	2.99 ± 0.29[d]
IMC-67	Catongo	2.15 ± 0.06[e]

[a] Mean of three sample preparations (duplicate injections of each preparation) ± standard deviation. Values followed by a common letter are not significantly different ($P \geqslant 0.05$) in Tukey's ω method of mean separation.

Results from another fermentation in a different country, Trinidad, showed similar trend (Fig. 2). The concentration of (-)-epicatechin in the cotyledon decreased as fermentation progressed.

Fermentation of cocoa beans is crucial for the development of precursor for chocolate flavor. Complex interactions among the polyphenols to form high molecular weight tannins and their interactions with proteins impact on the overall quality of fermented cocoa beans for chocolate production. The sharp decreases observed in Fig. 1. and 2 reflect the onset of these phenomena during fermentation.

Fermented cocoa beans of verified genetic origin from Brazil were analyzed for (-)-epicatechin content (Table 3). Since these beans were obtained from a research farm, post-harvest treatments were assumed to be controlled and similar for all batches. Except for the ICS-1 clone, concentrations (2–3 mg) of (-)-epicatechin were very low. The significantly higher concentration in ICS-1 might suggest a fermentation variable, although this could not be ascertained with any degree of certainty because specific details of the process are lacking. During drying after fermentation, considerable oxidation of (-)-epicatechin can occur (Griffith, 1957). However, if fermentation in this series of samples followed the pattern of almost complete (-)-epicatechin consumption (Fig. 1 and 2), little would be left to be influenced by a drying variable.

SUMMARY

COCOA BEANS of verified genetic origin, freeze-dried immediately after removal from the pods to represent freshly harvested beans unaffected by any post-harvest treatments, were analyzed for (-)-epicatechin content. Concentrations ranged from 36.4–43.2 mg with small variation among the samples. A significantly lower concentration (21.8 mg) in the Trinidad-Jamaican beans can be attributed to experimental microwave and sun-drying treatments after harvest. Amounts of (-)-epicatechin in commercial beans from different regions of production tended to be highest in beans from regions where fermentation practices are less extensive. Trinidad-Jamaican beans, analyzed at various stages of fermentation, showed a sharp decrease of (-)-epicatechin in the nibs between the second and third day of fermentation. Coinciding with this decrease, (-)-epicatechin content in the shells increased. Analysis of Trinidad beans fermented at a different location confirmed the trend towards a decrease in (-)-epicatechin content in the nibs as fermentation progressed. Except for the ICS-1 clone of the Trinidad-Jamaican beans, (-)-epicatechin content (2–3 mg) among fermented beans of known genetic origin was very low. Fermentation variables probably account for the higher (-)-epicatechin content (10.0 mg) in the ICS-1 clone.

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Hydrophobic Interaction in the Gelation of High Methoxyl Pectins

DAVID OAKENFULL and ALAN SCOTT

ABSTRACT

The role of hydrophobic interaction between the ester methyl groups in the gelation of high methoxyl pectins was investigated by using temperature and different cosolutes to modify hydrophobic interaction in a controlled manner. Both rupture strength and gel threshold were found to be partly proportional to the free energy of hydrophobic interaction between CH_3-groups in model systems. The size of junction zones and the standard free energy of gelation were proportional to the square of the degree of methoxylation, i.e. to the probability of ester methyl groups being opposed in contiguous lengths of polymer. The contribution from hydrophobic interaction to the free energy of gelation was about half that from hydrogen bonds.

INTRODUCTION

PECTINS have been used as gelling agents on a large scale (Glicksman, 1969) and over a long period of time (Braconnot, 1825) but the mechanism of gelation is not well understood at the molecular level (Rees, 1972). Pectins are linear polymers, consisting almost entirely of D-galacturonic acid. They are prepared commercially by partial acid hydrolysis of the "pectic substances" which are a major structural component of the cell walls of plants (Glicksman, 1969; Ahmed, 1981). The acid groups are partly esterified with methanol with the proportion esterified, the degree of methoxylation (DM), depending on the conditions during preparation.

The work reported here was with pectins of DM > 50% – "high methoxyl" pectins. These pectins form gels if the pH is below about 3.6 and if a cosolute is present, typically sucrose at a concentration of greater than 55% by weight (Glicksman, 1969; Ahmed, 1981).

The physical properties of a gel are the consequence of the formation of a three-dimensional network of polymer molecules (Flory, 1953). Thus the conditions required for gelation and the properties of the gel ultimately depend on the molecular structure, the intermolecular forces which hold the network together and the nature of the junction zones where the polymer molecules are cross-linked. The junction zones in polysaccharide gels are complex and the molecular structures are held together by a large number of individually weak interactions, such as electrostatic interactions and hydrogen bonds (Rees, 1969). Evidence from X-ray diffraction studies (Walkinshaw and Arnott, 1981) suggest that in gels of high methoxyl pectins the junction zones have the structure shown in Fig. 1. This structure would be stabilized by hydrogen bonds (indicated by dotted lines) and also by hydrophobic interaction of the ester methyl groups (indicated by filled circles).

The stability of hydrophobic interactions can be modified by adding different sugars or polyols (Back et al., 1979), ethanol, *t*-butanol or dioxane (Oakenfull and Fenwick, 1979) or by changing the temperature. We have used these means to investigate the role of hydrophobic interaction in the gelation of high methoxyl pectins, systematically varying the hydrophobic contribution to the process of gelation.

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MATERIALS & METHODS

Pectins

Three of the pectins were prepared from commercial materials [Copenhagen Pectin Factory Ltd and Davis-Germantown (Aust.) Ptd. Ltd] by removing, by means of dialysis, the sucrose which these contained. The degree of methoxylation was determined by titration (McCready, 1970). Intrinsic viscosities were measured with Ostwald viscometers which had flow times for water of about 20 sec. The degree of methoxylation of one of the pectin samples was increased by treatment with diazomethane (Hough and Theobald, 1963). The properties of the four pectins are summarized in Table 1.

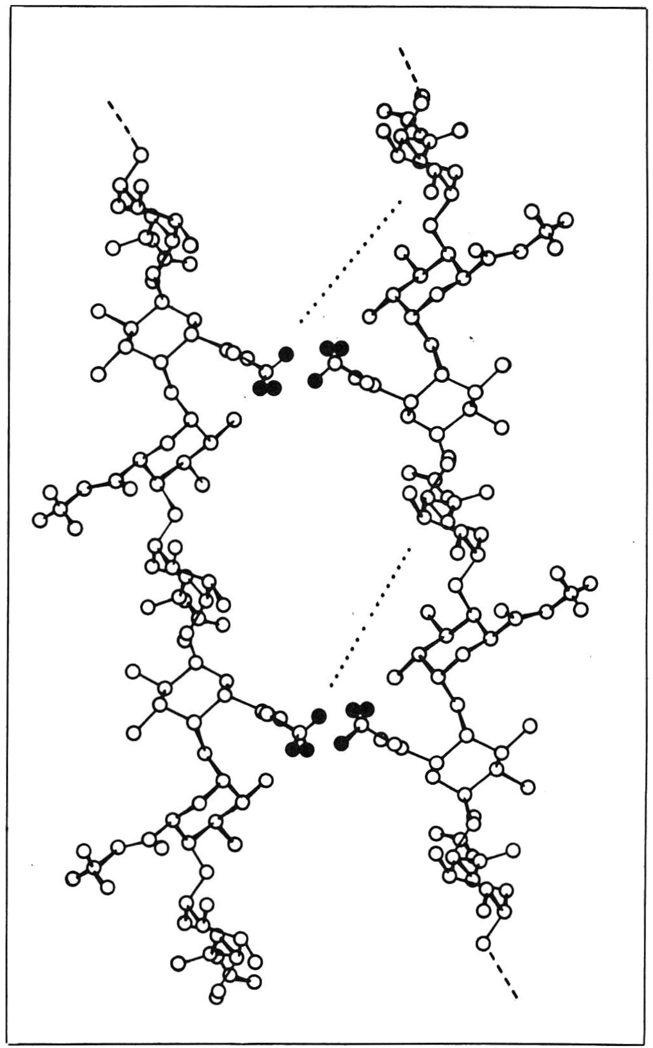

Fig. 1—Structure of junction zones in gels of high methoxyl pectins inferred from X-ray diffraction studies (Walkinshaw and Arnott, 1981).

Preparation of gels

The dry pectin was mixed first with the cosolute, such as sucrose or ethanol, and thoroughly dispersed. The appropriate weight of water was added and the mixture was placed in a sealed flask on a boiling water bath for about 1 hr and stirred occasionally during this time. (Prolonged but gentle heating was required at this stage to hydrate the pectin fully and thus obtain gels with reproducible properties.) Finally sufficient citric acid (0.11 mol L^{-1}) was added to give a concentration of citrate of 0.011 mol L^{-1}. The gels were routinely set at 25°C for 18 hr before meaurements were made.

Rheological measurements

Most measurements were made with an Instron Model 1122 Universal Testing Instrument. The gels were cast in cylindrical molds of diameter 4.0 cm and thickness 1.2 cm. Apparent shear moduli and rupture strengths were measured under compression with a cross-head speed of 5 mm/min. For very weak gels, of shear modulus <500 Nm^{-2}, the apparatus of Saunders and Ward (1954) was used. Increased sensitivity was obtained by replacing the mercury in the manometer with carbon tetrachloride.

A number of measurements of rupture strength were made at temperatures different from ambient temperature (20–25°C) using the "Single Puncture Maturometer" described by Huntington and Rutledge (1974). This instrument is portable and could be used in different constant temperature rooms within the range 0 - 50°C (±1°). The gels were set in crystallizing dishes of diameter 13.5 cm and the probe had a cross-sectional area of 4.52 cm^2. This apparatus gave values for rupture strength similar to those obtained using the Instron; typically, a gel containing 7.2 g/kg pectin B and 230 g/kg ethanol had rupture strength of 1.48 kNm^{-2} measured with the maturometer and 1.20 kNm^{-2} measured with the Instron.

Measurement of gel threshold

Solutions of appropriate composition, covering a range of concentrations of pectin or cosolute, were prepared in screw-capped flat bottomed scintillation vials of internal diameter 2.4 cm, 10g of solution to each vial. The solutions were allowed to equilibrate for 18 hr and the vials were then gently inverted. A solution which remained in position in the vial was considered to have gelled.

Table 1—Intrinsic viscosity[a] and degree of methoxylation of the pectin preparations

Pectin	DM (%)	[η] (mL g^{-1})
A	93.0	306
B	72.3	340
C	69.7	306
D	64.9	254

[a] Measured in 0.04 mol L^{-1} phosphate buffer at pH = 7.12, at 25°C.

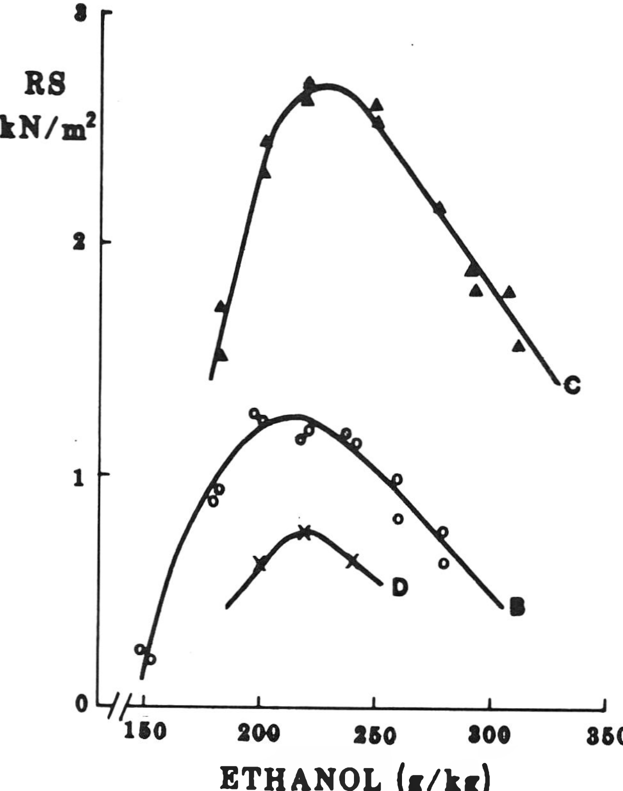

Fig. 2—Rupture strength (RS) of gels prepared from pectins B (3.3 g/kg), C (3.6 g/kg) and D (3.4 g/kg) in solutions containing ethanol.

RESULTS & DISCUSSION

Gels prepared with ethanol, t-butanol or dioxane

The sucrose usually used to prepare pectin gels can be replaced by dioxane or much smaller concentrations of ethanol or t-butanol. Fig. 2 and 3 show how the rupture strength of gels prepared with ethanol, *t*-butanol or dioxane as cosolute varies with the concentration of cosolute. These results appear to reflect the effects of the cosolutes on hydrophobic interaction between pairs of CH_3-groups. These effects have been investigated using model systems (Oakenfull and Fenwick, 1979). Dioxane weakened hydrophobic interaction, but small concentrations of ethanol or *t*-butanol strengthened hydrophobic interaction as shown in Fig. 4. The free energy of hydrophobic interaction between a pair of CH_3-groups (ΔG_{HI}) initially became more negative with the addition of alcohol, reached a minimum value and then increased (Oakenfull and Fenwick, 1979). For gels formed in the presence of ethanol or *t*-butanol, maximum rupture strength occurred at about the same concentration as maximum hydrophobic interaction. Dioxane affected gelation differently. A large concentration (>350 g/kg) of it was required for pectin to gel and gelation was presumably induced by the low dielectric constant which would stabilize polar interactions. A solution of 350 g/kg dioxane in water has a dielectric constant of 49.0 whereas the alcohol solutions that were studied had dielectric constants within the range 60–70 (Åkerlöf, 1932; Brown and Ives, 1962).

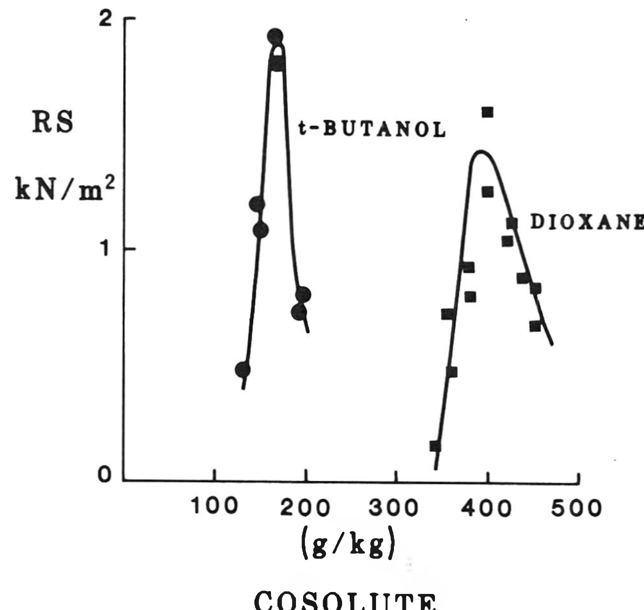

Fig. 3—Rupture strength (RS) of gels prepared from pectin C (3.6 g/kg) in solutions containing t-butanol (●) or dioxane (■).

There is, as yet, no satisfactory theoretical relationship between rupture strength and intermolecular forces (Mitchell, 1980). It is possible, though, to relate the gel threshold, or minimum concentration of polymer which will form a gel (C_m), to the thermodynamics of junction zone formation (Pouradier, 1967). The standard free energy of formation of junction zones (ΔG°_j) is related to C_m by the expression

$$\Delta G^\circ_j = Q + RT \ln C_m \quad (1)$$

where Q is a constant (see appendix). Fig. 5 shows how $RT \ln C_m$ varies with the concentration of ethanol. The experimental minimum value again coincide with maximum hydrophobic interaction.

The curve in Fig. 5 was calculated by assuming that the contribution to ΔG°_j from polar interactions was inversely proportional to the dielectric constant of the solvent (ϵ) and the hydrophobic contribution directly proportional to the free energy of hydrophobic interaction between a pair of CH_3-groups (ΔG°_{HI}). Thus

$$\Delta G^\circ_j = A + B\,\Delta G^\circ_{HI} + C/\epsilon \quad (2)$$

where A, B and C are constants. Values of ΔG°_{HI} were taken from Oakenfull and Fenwick (1979) and of ϵ from Åkerlöf (1932). The three constants could then be estimated by multiple linear regression. The values obtained were: A = 63.5, B = 8.60 and C = −3170. The coefficient of regression was 0.877.

Since the value of B is the effective number of CH_3-CH_3 hydrophobic interactions per junction zone it is possible to calculate the lower limit of the mean size of the junction zones from this result. The pectin was 69.7% esterified, consequently, if the esterified glucuronic acid units are randomly distributed along the polymer chain, the probability of two methyl groups being adjacent in a junction zone is $(0.697)^2$. Thus the 8.6 pairs of CH_3-group interactions per junction zone would occur if the junction zones consisted of two strands of polymer, each containing an average of $8.6/(0.697)^2 = 18$ monomer units. However, the junction zones are likely to be larger than this since the chains have limited flexibility and the methyl-groups of adjacent esterified galacturonic acid units need not necessarily be close enough together for hydrophobic interaction to occur.

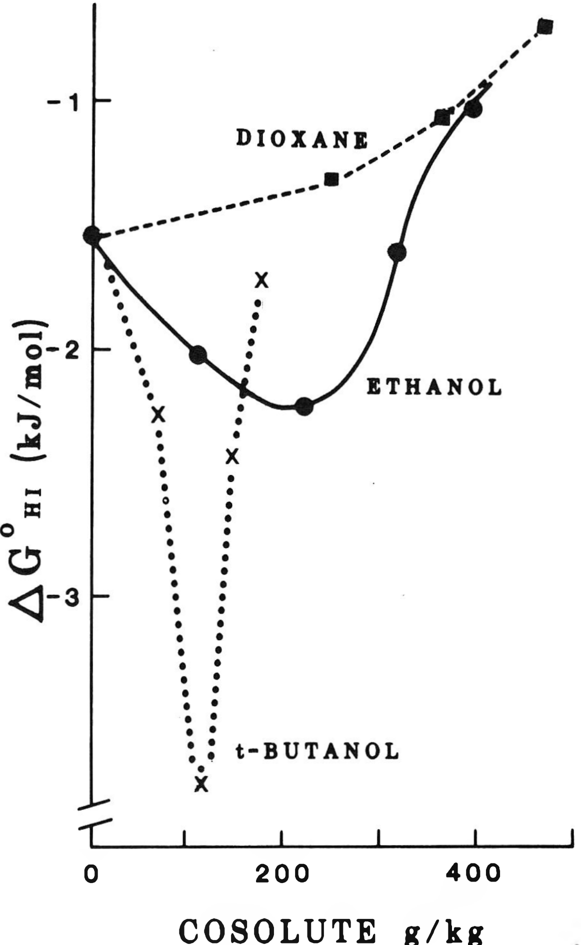

Fig. 4—Standard free energy of hydrophobic interaction (ΔG°_{HI}) plotted against the mole fraction in water of ethanol (●), t-butanol (x) and dioxane (■), at 25°C (from Oakenfull and Fenwick, 1979).

Gels prepared with sugars or polyols

The sucrose usually used to prepare gels of high methoxyl pectins can be replaced by a number of other sugars or polyols, such as glucose, fructose, or glycerol (Deuel et al., 1953). Sugars and polyols can stabilize hydrophobic interactions and it has been shown that this effect explains the increased thermal stability of globular proteins in the presence of certain sugars or polyols (Back et al., 1979). The magnitude of the effect depends on the stereochemistry of the sugar or polyol since the spacing and orientation of the hydroxyl-groups determine how these compounds interact with water (Franks et al., 1972; Tait et al., 1972).

We have determined a series of concentrations of sugars and polyols which give equal free energies of junction zone formation for pectin C. This was done by measuring the minimum concentration of sugar or polyol which would induce gelation of a solution containing 3.6 g/kg of the pectin at 298 K. These results appear to correlate (coefficient of regression = 0.812) with the effect of the appropriate sugar or polyol on the denaturation temperature of ovalbumin (ΔT_m) as reported by Back et al. (1979). The term ΔT_m is the difference between the denaturation temperature in aqueous buffer and that in the presence of 500 g/kg sugar or polyol; it can be used as an empirical measure of stabilizing effect of the sugar or polyol on hydrophobic interaction. This finding suggests that the function of sugar

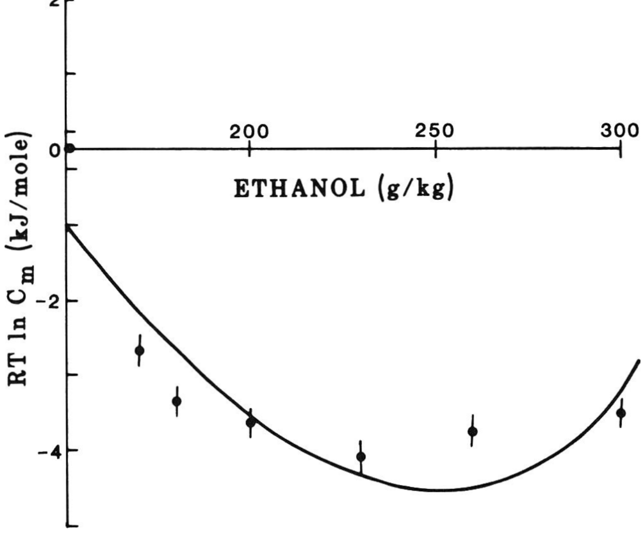

Fig. 5—RT ln C_m plotted against the concentration of ethanol. The curve was calculated from Eq. (2) as described in the text.

in the formation of gels of high methoxyl pectins is to stabilize junction zones by promoting hydrophobic interaction between ester methyl groups. The effect thus depends specifically on the molecular geometry of the sugar and the interaction with neighboring water molecules. Chen and Joslyn (1967) have similarly found specific effects of sugars on the association of pectin molecules, but in solutions too dilute for gelation. They found that added sucrose, glucose or maltose increased the reduced viscosity of these solutions, whereas dextrins lowered it. and that glucose and sucrose had a greater effect than maltose. There is no support from these two sets of results for the traditional view that sugar acts nonspecifically by partly dehydrating the pectin molecules (Goldthwaite, 1909; Rees, 1969). If this theory were correct then solutions of sugar or polyol at the minimum concentration required for gelation would have equal water activity. The data presented in Table 2 show that this is not the case.

Effect of temperature on gel strength

The change of rupture strength with temperature also indicates that high methoxyl pectin gels are stabilized by hydrophobic interactions. Rupture strengths of gels prepared from a fixed concentration (3.6 g/kg) of pectin C were measured at temperatures within the range 0 – 50°C. (The gels were equilibrated at the temperature of the measurements.) The results are presented in Fig. 6 which includes, for comparison, some results of equivalent experiments carried out with κ-carrageenan. The curve for the pectin is sigmoidal, with a small peak at ~30°, whereas the rupture strength of the carrageenan gels decreased monotonically with increasing temperature. This result is explained qualitatively by the opposing effects of increasing temperature on hydrogen bonding and hydrophobic interactions. Hydrogen bonds are weakened by increasing temperature (Joesten and Schaad, 1974) whereas, within the temperature range 0 – 50°C hydrophobic interactions become stronger with increasing temperature (Oakenfull and Fenwick, 1977; Ben-Naim, 1980). These competing effects could generate a curve of the form of that for the pectin in Fig. 6. Such a complex response to temperature has not been reported for other polysaccharide gels but a maximum in rupture strength vs temperature for pectin has also been reported by Deuel et al. (1953).

Table 2—Values of minimum concentration for gelation of pectin C, corresponding water activity and extent of thermal stabilization of a series of sugars and polyols

Sugar or polyol	C_m (g kg^{-1})	a_w [a]	ΔT_d [b] (°C)
Sorbitol	390	0.934	14.0
Glucose	400	0.932	15.5
Xylitol	400	–	13.0
Ribitol	450	–	11.5
Maltose	450	–	10.0
Fructose	460	0.912	12.0
Sucrose	470	0.944	11.0

[a] Water activity at a concentration of solute of C_m at 25°C from Norrish (1966).
[b] Increase in the temperature of denaturation of ovalbumin in response to added sugar or polyol at a concentration of 500g kg^{-1} (Back et al., 1979).

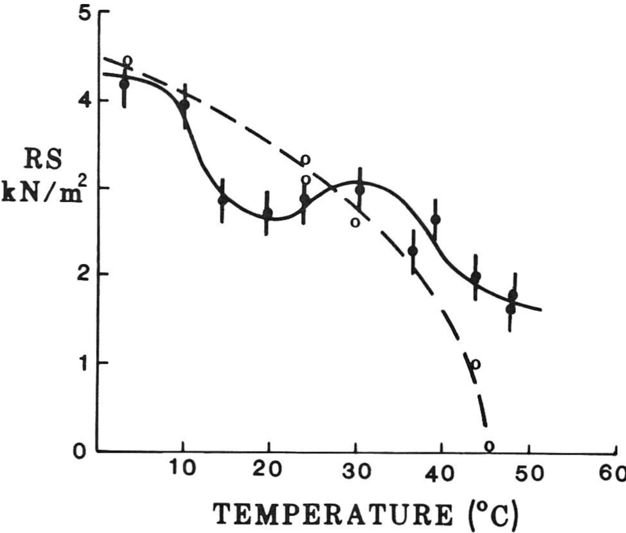

Fig. 6—Effect of temperature on the rupture strength of gels. Solid curve: 3.6 g/kg pectin C gelled with 550 g/kg sucrose. Broken curve: 10 g/kg κ-carrageenan.

Effect of degree of methoxylation on the size and stability of junction zones

The association constant (K_j) for the formation of junction zones and their number average molecular weight (M_j) can be estimated from measurements of shear modulus at concentrations close to the gel threshold (Oakenfull, 1984). The results of a series of such measurements for gels prepared from the four pectins A, B, C and D are shown in Fig. 7 (these gels all contained 550 g/kg sucrose). From these data we calculated values for K_j and M_j and also the number average molecular weight of the polymer (M) and the number of cross-linking loci per junction zone (n) (Table 3).

In each case the number of cross-linking loci per junction zone was close to two, which is consistent with model building studies (Rees and Wight, 1971) and the polymer molecular weights were within the range usually reported for commercial pectin preparations (Jordan and Brant, 1978).

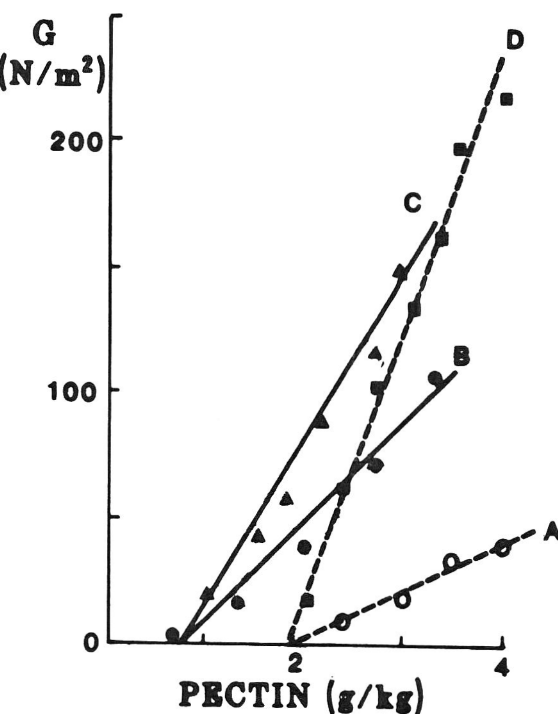

Fig. 7—Shear modulus at 25°C of pectin gels at concentrations close to the gel threshold. The gels contained 550 g/kg sucrose.

With the exception of the results for pectin A, which was almost fully methoxylated, Mj and the standard free energy of formation of junction zones ($\Delta G^\circ_j = -RT \ln K_j$) were proportional to the square of the degree of methoxylation (Fig. 8). The size and thermodynamic stability of the junction zones was thus proportional to the probability of hydrophobic interaction between opposing ester methyl groups in adjacent lengths of polymer. The highly esterified pectin A appeared to give junction zones of maximum size, with complete overlap of the polymer chains ($M_j = M$) as shown schematically in Fig. 9.

The value of M_j for pectin C was equivalent to 57 monomer units, considerably greater than the junction zone size of 36 monomer units derived from the effect of ethanol on the gel threshold. Presumably even when esterified galacturonic acid groups were appropriately aligned, configurational constraints may prevent the methyl groups from coming sufficiently close together for effective hydrophobic interaction. It is possible to use the data given in Table 3 to estimate the fraction of aligned esterified galacturonic acid groups which effectively contributed hydrophobic stabilization to the junction zones.

The standard free energy of formation of a junction zone consists of contributions from hydrophobic interaction, hydrogen bonding, and the loss of configurational entropy of the polymer chains. Thus,

$$\Delta G^\circ_j = (p/2)\, e^2 f\, \Delta G^\circ_{HI} + (p/s)\, \Delta G^\circ_{HB} - pT\Delta S_{conf} \quad (3)$$

where p is the number of monomer units per junction zone, e the fraction of esterified glucuronic acid groups, f the fraction of aligned esterified galacturonic acid groups which contribute hydrophobic stabilisation, ΔG°_{HI} the standard free energy of hydrophobic interaction of a pair of CH_3-groups [−2.34 kJ/mole (Back et al., 1979)], ΔG°_{HB} is the net hydrogen bonded interaction per pair of monomer units and ΔS_{conf} is the change of configurational entropy and is proportional to $\ell n\ p$ (Flory, 1953). Consequently, the free energy change per monomer unit,

$$\Delta G^\circ_j/p = 1.17 e^2 f + 0.5\, \Delta G^\circ_{HB} - A\, \ell n\ p \quad (4)$$

where A is a constant. The three sets of values of e, p and ΔG°_j from Table 3 then give f = 0.57, ΔG°_{HB} = 1.31 kJ/mole and A = 0.177. If the factor f is included in the calculation of the size of junction zones from the effect of ethanol on the gel threshold, the result becomes 36/0.57 = 63 monomer units which is close to the result from shear modulus measurements given in Table 3.

More importantly, these calculations indicate the relative contributions to the stabilization of junction zones from hydrophobic interactions and hydrogen bonds. Considering pectin C as a typical example, the hydrophobic term in Eq. (3) has the value −18.6 kJ/mole, the hydrogen bonding term is −37.5 kJ/mole and the change in configurational entropy is equivalent, in terms of free energy, to 41.1 kJ/mole. The contribution from hydrogen bonding is twice that from hydrophobic interactions but hydrogen bonding alone is insufficient to stabilise the junction zones.

CONCLUSIONS

THE NETWORK of polysaccharide molecules in gels of high methoxyl pectins is stabilized by a combination of hydrophobic interactions and hydrogen bonds. The contribution from hydrophobic interaction to the free energy of formation of junction zones is half that from hydrogen bonding but is an essential requirement since hydrogen bonding alone is insufficient to overcome the entropic barrier to gelation.

Junction zones consist of two adjacent segments of polysaccharide chain varying in length from 18 to about 250 galacturonic acid units, increasing with the degree of methoxylation.

The requirement for a high concentration of sucrose (or other similar cosolute) in the preparation of pectin gels is explained by the stabilizaton of hydrophobic interactions by certain sugars (including sucrose). The significant role of hydrophobic interaction may also explain how the rheological properties of these gels depend on temperature and are affected by the substitution of other sugars or similar cosolutes for sucrose.

Table 3—Number average molecular weights of the polymer and characteristics of the junction zones calculated from measurements of shear modulus of pectin gels containing 550g kg⁻¹ sucrose

Pectin	M[a]	M_J[b]	No. of monomer units/junction zone	K_J[c]	n[d]
A	92 000	92 000	497	–	2.01
B	249 000	25 000	135	1380	2.08
C	113 000	10 600	57	420	2.03
D	38 000	6 230	34	70	1.97

[a] Number average molecular weight of the polymer.
[b] Number average molecular weight of the junction zones.
[c] Association constant for the formation of junction zones; in units of $L\ mol^{-1}$.
[d] Number of cross-linking loci per junction zone.

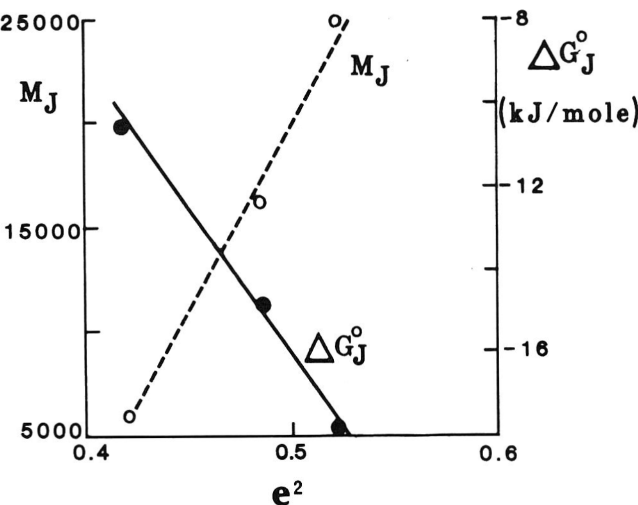

Fig. 8—Number average molecular weight of junction zones (M_j) and the standard free energy of formation of junction zones (ΔG°_j) plotted against the square of the degree of methoxylation (e^2).

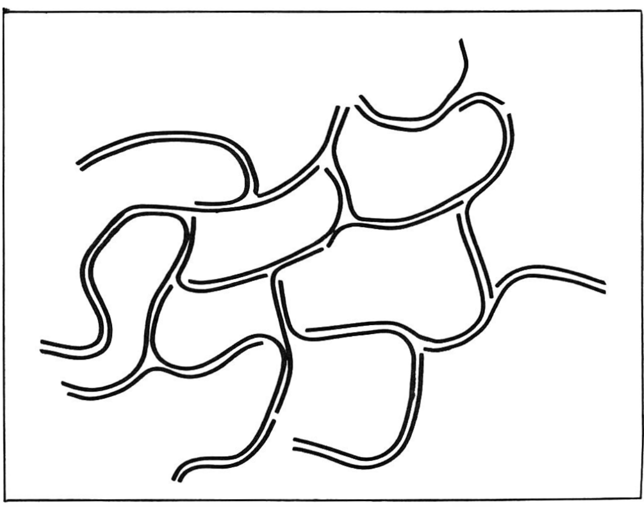

Fig. 9— Schematic diagram of a gel network with maximum overlap of the polymer chains.

APPENDIX

Relationship between gel threshold and the thermodynamics of junction zone formation

At the gel threshold there is a certain critical number j of junction zones per polymer molecule (Flory, 1953; Pouradier, 1967). The value of j must remain arbitrary since the gel threshold is itself arbitrarily defined (by whether or not the gel remains fixed in position in a tube on inversion). If C_m is the gel threshold, the minimum concentration of polymer that will form a gel, the number of junction zones per unit volume,

$$J = j\, N_{av} C_m / M \qquad (1)$$

where N_{av} is Avogadro's number and M is the number average molecular weight of the polymer. Assume now that there are an average of ℓ cross-linking loci per polymer molecule, then the number of such loci per unit volume of gel is

$$L = \ell\, N_{av} C_m / M \qquad (2)$$

If n of these loci are required to form a junction zone, then by the law of mass action, the association constant

$$K = J/L^n = j\,(N_{av} C_m / M)^{1-n} / \ell^n \qquad (3)$$

There is evidence that in the case of pectin gels the junction zones consist of segments from only two polymer molecules, i.e. n = 2 (Rees and Wight, 1971; Oakenfull, 1984). Thus

$$K = (jM / N_{av} \ell^2)\,.\, 1/C_m \qquad (4)$$

so that the standard free energy of formation of junction zones is given by

$$\Delta G^{\circ}j = -RT\, \ell n\, K = RT \ln C_m + \text{constant} \qquad (5)$$

This treatment assumes, of course, that gelation is an equilibrium process, subject to the law of mass action. This assumption and the limitations it imposes are discussed in a previous paper (Oakenfull, 1984).

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A Comparison Between Ovalbumin Gels Formed by Heat and by Guanidinium Hydrochloride Denaturation

B. EGELANDSDAL

ABSTRACT

Ovalbumin was denatured by heat or by addition of 6M guanidinium hydrochloride (GuHCl) at pH 2.5. Denaturation by heat led to immediate gel formation whereas denaturation by GuHCl yielded gels only after subsequent removal of the denaturant. The two types of gels formed were compared by using texture measurements. Chemically denatured molecules had the largest hydrodynamic volume and also formed the hardest gels (protein concentration: 6% w/v). This difference in gel hardness was, however, much enhanced if the denaturant was slowly removed from the protein solution. Hence, the rate of gel formation appears to be of greater consequence than the degree of protein unfolding for the hardness of the gels formed.

INTRODUCTION

GLOBULAR PROTEINS do not possess good heat-gelling properties compared to gelatin and other fibrous proteins (Bezrukov *et al.*, 1978). Substantial protein concentrations are therefore required to obtain three-dimensional networks. The type of network formed varies considerably, both at short and long distances, depending largely upon pH and ionic strength. At high net protein charges and low ionic strengths clear and uniform gels are formed by directed, linear aggregation (Tombs, 1974; Clark *et al.*, 1981). More irregular networks formed by essentially random aggregation are the case under conditions of low electrostatic repulsion between individual polypeptide chains. The degree of randomness upon agregation and the texture of the resulting gels are surmised to depend upon the extent of protein unfolding (Clark *et al.*, 1981). Ovalbumin has been suggested to unfold only marginally upon heat treatment (Holme, 1963; Smith, 1964). Heating at about pH 2.5 is assumed to maximize the limited thermal unfolding of the protein due to the high net charge of the protein at this pH (Barbu and Joly, 1952; Foster and Rhees, 1952). In contrast, denaturation by concentrated (6M) solutions of guanidinium hydrochloride (GuHCl) containing 0.1M 2-mercaptoethanol (2-ME) yields essentially unfolded ovalbumin molecules (Ansari *et al.*, 1972; Ahmad and Salahuddin, 1974).

In this study, gelation of ovalbumin was induced by heat-treatment and by GuHCl denaturation and the effect of the extent of protein unfolding and rate of aggregation on the gels formed was investigated. The objective of the investigation was two-fold: to increase the fundamental knowledge of the factors that govern gel formation, and to provide information about the properties of globular proteins.

MATERIALS & METHODS

OVALBUMIN was isolated from fresh eggs by the method of Rhodes *et al.* (1958).

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Determination of protein concentrations

Protein concentrations in solutions were determined from their light absorbance at 280 nm, assuming $E_{1\,cm}^{1\%} = 7.12$ (McKenzie *et al.*, 1963). To determine concentrations in gels, pre-weighed gels were solubilized in known amounts of 6M GuHCl containing 0.1M 2-ME. The concentration of protein in the resulting solution was determined by the protein-dye binding method of Bradford (1976).

Intrinsic viscosity measurements

These were performed with a Cannon-Ubbelohde semi-micro viscometer at 54°C on solutions at pH 2.5. For each concentration of protein the reduced viscosity was determined four times on independently prepared solutions. Diluted solutions were prepared from the most concentrated one (1% w/v) by diluting prior to heat treatment with an acid solution (pH 2.5) containing 0.006M NaCl in order to simulate isoionic dilution (Tanford, 1961). This concentration of NaCl corresponds to the concentration of HCl in an ovalbumin solution (1% w/v) obtained by titration of the protein from its isoelectric state (pH = 4.6) to pH 2.5.

Intrinsic visocity $[\eta]$ was determined by extrapolation to zero protein concentration using a straight line curve fitting computer program. In the plot of $[\eta]$ as a function of time (Fig. 1), the times reported are from the moment when heat-treatment started till the solution had flowed half-way through the viscometer.

Preparation of chemically induced gels

Freeze-dried protein was weighed out and dissolved in an aqueous solution of pH 2.5, containing 6M GuHCl and 0.1M 2-ME, to give approximately the desired protein concentration. The actual concentration in the gels was determined as described above. The protein solution was stored overnight at about 22°C to equilibrate the mixture. Aliquots (2 mL) of the solution were pipetted into dialyzing bags (diam. 1.6 cm) tightly fitted with polytetrafluoroethylene cylinders at their lower ends. The bags were closed in such a manner as to produce a slightly enhanced internal pressure relative to the ambient pressure. Finally, the bags were fixed in an up-right position in the dialyzing solution.

Dialysis technique 1. Each bag was dialyzed for 24 hr against 200 mL of a stirred solution of pH 2.5 containing fixed concentrations of GuHCl (0-4.5M) and 2-ME (0-0.075M). The ratio between the two denaturants was, however, always kept constant.

Dialysis technique 2. Each bag was positioned inside a cell made from a socket and a cone adaptor (size 34/35, Quickfit). One liter of solution (pH 2.5 or 4.6) continuously changing from 6M GuHCl and 0.1M 2-ME to 0M GuHCl and 0M 2-ME, respectively, was pumped through the cell in the course of 2-22 hr. Subsequently, 3L of hydrochloric acid (pH 2.5 or 4.6) were passed through the cell. The bags remained in the cells for a total of 24 hr. In an exception to the general procedure the gradient was pumped through the cell during the course of 47 hr and the hydrochloric acid in 3 hr. Denaturant concentrations in the dialysate/effluent were monitored by continuous UV-measurements at 260 nm. This dialysis technique reduced the concentrations of GuHCl inside the bags to less than 10^{-4}M as determined by conductivity measurements on a dummy solution.

Preparation of heat induced gels

The protein was dissolved in distilled water or in a solution of NaCl to give 6% (w/v). The pH was adjusted to 2.5 (or 4.6) using 0.5M HCl and aliquots of the solutions were transferred to the same type of dialysis bags as described above. The bags were heated for 40 min at a constant temperature between 54° and 69°C in a bath of the protein solvent and then stored 24 hr at about 22°C submerged in the same solvent.

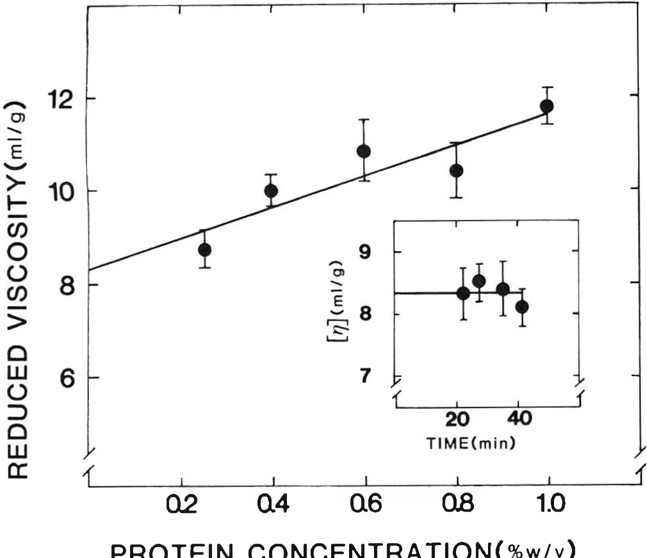

Fig. 1—Reduced viscosity of ovalbumin diluted isoionically from 1% (w/v) as a function of protein concentration at pH 2.5 and 54°C. The samples were heat-treated for 21 min. The insert shows the intrinsic viscosity as a function of time of heating in 0.006M NaCl. The bars indicate S.E. (4 observations).

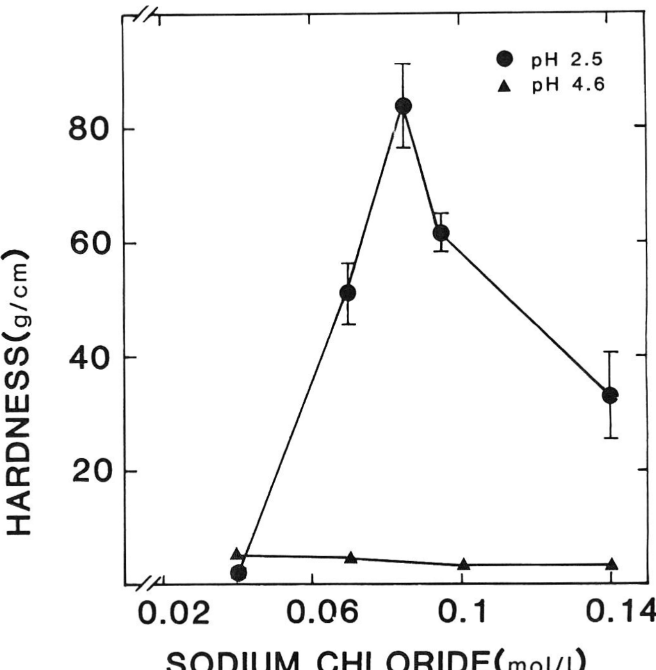

Fig. 2—Hardness of 6% (w/v) ovalbumin gels as a function of the concentration of NaCl. The gels were heat-induced at 54°C (pH 2.5) and 69°C (pH 4.6). The bars give S.E. (4 observations).

Determination of hardness

The cylindrical gels obtained were shaped to heights of 0.9 cm. At about 22°C these were indented in the middle and near the edge at 0.25 cm/min by a cylindrical plunger (0.45 cm) attached to an Instron Universal Testing Machine (Model TMSM). The mean initial slope oft the force-deformation curve is for each sample tested, reported as hardness.

RESULTS

Estimation of the intrinsic viscosity of the heat-denatured protein molecules

The reduced viscosities determined at pH 2.5 and at 54°C are shown in Fig. 1. The straight line fitted to the measured points in the main figure (r^2 = 0.82) gives the intrinsic viscosity at the intersection with the ordinate. As seen from the insert $[\eta]$ did not show any significant time-dependency, being 8.3 ± 0.4 ml/g (estimated S.E.). The nonheated protein was found to have $[\eta]$ = 4.1 mL/g at 20°C.

The extent of denaturation after heating for 21 min at 54°C was complete (≥96%) as determined both by isoelectric precipitation (Smith, 1964) and by differential scanning calorimetry (Hegg *et al.*, 1979).

Hardness of heat-induced gels

Fig. 2 shows the hardness of heat-induced gels at pH 2.5 and 4.6 as a function of the concentration of NaCl. Gel hardness at pH 4.6 was consistently very small while gels at pH 2.5 displayed strong dependence on ionic strength. At the latter pH there was a gradual change from transparent gels to coagulates with increasing concentrations of salt whereas only coagulates were formed at pH 4.6.

The largest values for hardness obtained, were, as shown in Fig. 2, 84 g/cm (in 0.085M NaCl) and 7 g/cm at pH 2.5 and 4.6, respectively.

Effect of temperature of heat-treatment on hardness of heat-induced gels

Gel hardness was essentially independent of the temperature at which the protein solutions were heat-treated (Fig. 3).

Thermal treatment for 40 min at temperatures above 65°C was deemed futile due to hydrolysis (Vaag, 1982).

Effect of final denaturant concentration (dialysis technique 1) on hardness of gels

After dialyzing the protein initially in 6M GuHCl and 0.1M 2-ME against different and lower concentrations of denaturants, the data shown in Fig. 4 were obtained. The hardness increased for decreasing equilibrium concentrations of denaturants and also for a decreasing protein concentration in the gels. The relationship between hardness (h) and protein concentration (C_p) was found to be $h \propto C_p^{1.4}$ for all the concentrations of denaturants investigated. This relationship was used to normalize the hardness data whenever the results for gels at a given protein concentration were desired.

The gels formed in 4.5M GuHCl were all transparent. At 4M and at lower concentrations of GuHCl the gels were opaque.

For chemically induced gels containing 6% (w/v) protein, the maximum value for hardness measured was 117 g/cm. Extrapolation of curve b (Fig. 4) to zero concentrations of the denaturants gave the value 118 ± 8 g/cm (estimated S.E.) for hardness. This value was significantly (5% level) higher than the maximum value obtained by any heat-treatment.

Effect of rate of aggregation (dialysis technique 2) on the hardness of chemically induced gels

Denaturant concentration in the solution outside the dialyzing bags as a function of time is shown in Fig. 5 for a series of pumping rates, *i.e.* a series differing in the total time allowed for the concentration of denaturants to decrease from the original values (6M GuHCl and 0.1M 2-ME) to nil. The different total times employed to complete the gradient were estimated by extrapolation of the curves a-f to zero absorbance by the dialysate; the extrapolated parts (Fig. 5) start where the curves are discontinued at their lower ends. The extrapolation was carried out because the lower ends of the curves tended to deviate from the shape

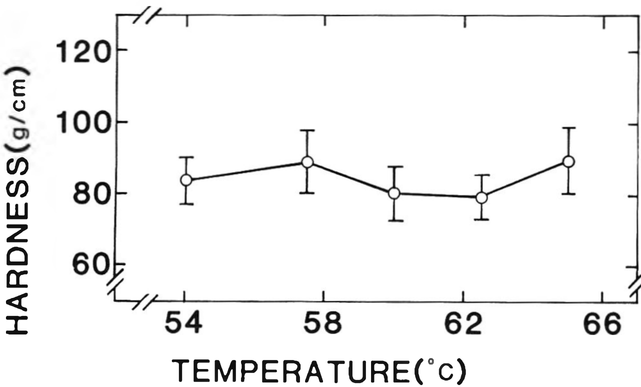

Fig. 3—Hardness of 6% (w/v) ovalbumin gels containing 0.085M NaCl (pH 2.5) as a function of the temperature of heat-treatment. The bars give S.E. (4 observations).

of the major, upper parts. The total times of dialysis against gradient constitute the abscissas of the points plotted in Fig. 6. A linear relationship was found between time allowed for dialysis and gel hardness. It is noteworthy that the values for hardness reported in Fig. 6 are all higher than any value for heat-induced gels at the same protein concentration. The maximum values for hardness obtained after dialysis for 24 hr, were 247 g/cm at pH 2.5 and 163 g/cm at pH 4.6 (Fig. 6, e_1 and e_2). The minimum values for hardness obtained by linear extrapolation to zero time of dialysis, was 144 ± 5 g/cm (estimated S.E.). The gel formed after approximately 2 days of dialysis (Fig. 6, f) was even harder than the maximum value reported above. However, this gel was not strictly comparable to those formed after 1 day only since this gel experienced a longer time of storage. All gels referred to in Fig. 6 were opaque.

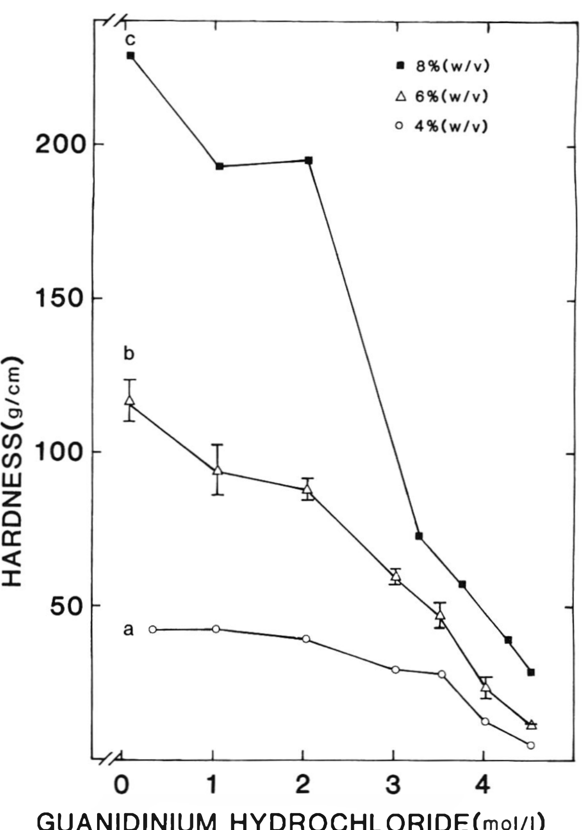

Fig. 4—Hardness of chemically induced ovalbumin gels (pH 2.5) as a function of GuHCl concentration. The protein was denatured in 6M GuHCl and 0.1M 2-ME prior to dialysis to the denaturant concentrations given. The bars give S.E. (3 observations).

DISCUSSION

HEAT-DENATURED OVALBUMIN is highly prone to aggregation (Holme, 1963). This phenomenon constitutes an element of uncertainty when deducing the conformation of heat-denatured ovalbumin molecules from hydrodynamic measurements. The dependency of the intrinsic viscosity upon time (Fig. 1) was therefore investigated in order to reveal possible effects of aggregation.

Within the time span relevant for the present determination, aggregation was found to be of no consequence. The value obtained for the intrinsic viscosity of the heat-denatured molecule was approximately twice the value of the native protein.

The intrinsic viscosity of ovalbumin in solutions of 6M GuHCl and 0.1M 2-ME has been determined by Ahmad and Salahuddin (1974). The latter authors gave the intrinsic viscosities as 20 mL/g at 54°C and 33 mL/g at 20°C. The values for $[\eta]$ at lower temperatures were theoretically in accordance with a random coil model (Ansari *et al.*, 1972).

Since the intrinsic viscosity of the heat-denatured molecules was found to be 8.3 mL/g at 54°C it is significantly smaller than the reported value for the chemically denatured molecules. This suggests that the molecules entering into the gels are fairly compact and may be characterized as highly hydrated ellipsoids/spheres rather than random coils.

A hypothesis could be put forward that the increased degree of unfolding reflected in the larger intrinsic viscosity is the sole reason for chemically denatured molecules giving stronger gels. A protein transformed to an expanded state before aggregation is induced, should be able to form a larger number of intermolecular contacts per polypeptide chain than compact molecules, thereby creating a more rigid network. Whether this is true or not can be investigated by comparing gels made from heat and chemically denatured proteins. The comparison should be applied to gels formed at the same rate of aggregation. In this study gels formed at the highest possible rate of aggregation have been compared.

For chemically denatured ovalbumin the hardness of gels can be determined from Fig. 4 and 6 by extrapolation to zero concentration of denaturants and zero time of dialysis against gradient, respectively. The values thus arrived at, 118 and 144 g/cm, expected to be close in magnitude, are not significantly different at the 5% level. Since it is conceivable that microheterogeneities may occur due to internal gradients of denaturant concentration during formation of gels produced by dialysis technique 1, the observed difference in hardness values is not surprising.

For heat-induced gels, high temperatures of heat-treatment favor fast aggregation. This is caused by the fact that aggregation closely follows denaturation and the rate of denaturation increases rapidly with increasing temperatures. However, no significant effect of increasing the temperature of heat-treatment was found in the temperature range investigated (Fig. 3). Thus the lower hardness value determined for gels made from thermally denatured proteins, 84 g/cm, suggests that chemically denatured proteins always will form harder gels, *i.e.* that the extent of protein unfolding is of some importance.

The comparison has so far been made at different ionic strengths, i.e. the hardness of chemically induced gels at zero salt concentration and that of heat-induced gels at 0.085M NaCl. As is seen from Fig. 2 the latter concentration of salt yields the strongest heat-induced gels. Fig. 4 shows that at low values for ionic strength, it has a limited importance for gels produced from chemically denatured

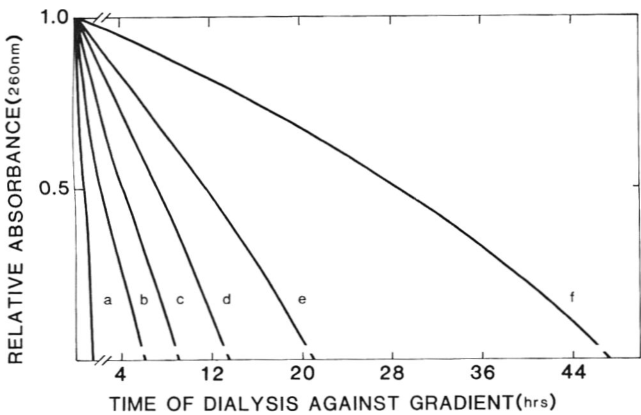

Fig. 5—Time dependent reduction in the concentration of GuHCl and 2-ME outside the dialyzing bags containing the protein.

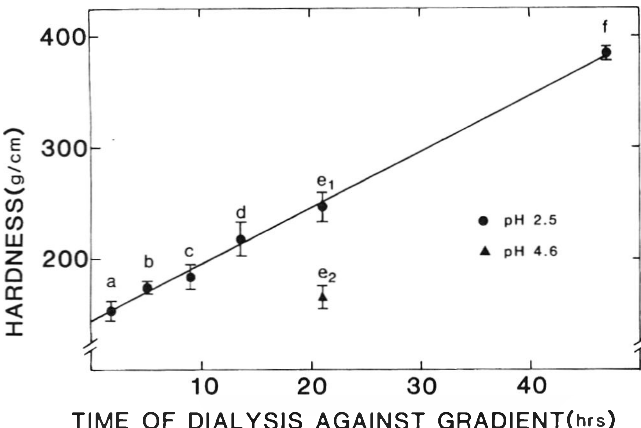

Fig. 6—Hardness of ovalbumin gels (6% w/v) obtained by simultaneously reducing the concentration of GuHCl and 2-ME outside the dialyzing bags from 6-0M GuHCl and 0.1-0M 2-ME, respectively. The abscissas plotted were obtained by extrapolation of the corresponding curves (a-f) in Fig. 5. The bars give S.E. (4 observations).

proteins. Thus if the above comparison is made at an ionic strength of 0.085M for both types of gels, the same conclusion is arrived at. It must, however, be emphasized that the suggested effect of unfolding upon hardness of gels is, in any case, quite small.

Higher ionic strengths, whether attained by a neutral salt or by GuHCl, prevent the formation of strong gels. The former component most likely by causing too fast aggregation, the latter due to rendering the protein chain highly soluble and thus preventing sufficient interactions between chains. However, under conditions where heat-induced aggregation produces weak gels at *low* ionic strengths, chemically induced aggregation is superior at producing strong gels. This is exemplified here at pH 2.5 and 4.6 (Fig. 4 and 6).

The rate of aggregation appears to have a dramatic effect on the hardness of the resulting gels. Fig. 6 strongly suggests that the longer the times given for the formation of physical bonds between the molecules, the stronger the gels formed. The reason for this may be the following: when the initial stage of gel formation proceeds slowly, time is allowed for reversible associations and dissociations to take place. Thus, molecules which initially form sub-optimal contacts have the time to dissociate and subsequently establish contacts with a more favorable free energy of interaction. These early formed points of strong interaction are very important, developing in the course of the aggregation process into irreversible bonds. The implication is that weak gels result from aggregation which is to a larger extent kinetically, as opposed to thermodynamically, controlled.

The effects of refolding of the protein chain upon the texture of gels have not been discussed so far. Refolding, even though theoretically possible, is assumed to be much hampered at the high protein concentrations employed here. Besides, even under quite favourable conditions, complete refolding of this protein is hardly possible (Egelandsdal, 1981). Unfortunately, this makes it difficult to obtain an unambiguous test for different and irreversible changes in the protein chain caused by the two denaturation methods used here.

This investigation elucidates the problems encountered in the production of gels from globular proteins. There is limited unfolding of the denatured protein in the aqueous solutions employed and too fast aggregation. The texture of heat-set protein gels is determined once the pH, the ionic strength and the temperature of heat-treatment have been fixed. The parameters like degree of unfolding and rate of aggregation can hardly be manipulated and an optimization with respect to the formation of strong gels is never achieved.

From a pracitcal point of view, the use of a chemical denaturant is of limited interest. Some more useful implications can, however, be deduced from this study.

Proteins of high net charge heat-denatured in solutions of low ionic strength, should produce stronger gels upon the slow administration of a neutral salt. This should be the case for heat-denatured ovalbumin molecules in a salt-free solution at pH 2.5 (Fig. 2). In addition, the texture of strong gels, once formed, is largely irreversible with respect to later changes in the composition of the imbibed aqueous solution.

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A Method for using Measurements of Shear Modulus to Estimate the Size and Thermodynamic Stability of Junction Zones in Noncovalently Cross-Linked Gels

DAVID OAKENFULL

ABSTRACT

A set of equations has been derived from which it is possible to calculate from measurements of shear modulus for noncovalent gels the association constant for the formation of junction zones, the number average molecular weight of the junction zones and the number of cross-linking loci per junction zone. It is assumed that the gels obey the theory of rubber elasticity and that the formation of junction zones is an equilibrium process subject to the law of mass action. The treatment is consequently valid only for very dilute gels at concentrations close to the gel threshold.

INTRODUCTION

THE PHYSICAL CHARACTERISTICS of a gel are the consequence of the formation of a continuous, three-dimensional, network of cross-linked polymeric molecules (Flory, 1953). In order to understand more about the relationship between the bulk rheological properties of gels and the molecular structure of gel-forming polymers it is necessary to know more about how these cross-linkages occur. We are concerned here specifically with noncovalently cross-linked gels in which the intermolecular forces are the relatively weak hydrogen bonding, electrostatic or hydrophobic interactions. A high degree of cooperativity is required and these cross-linkages may involve a number of monomer units which together form a "junction zone" (Rees, 1969). Spectroscopic studies have provided considerable insight into the nature of these junction zones (Rees, 1969; 1972). In the formation of gelatin gels, for example, the strands of protein are linked by a mutual helical structure as shown schematically in Fig. 1 (Harrington and von Hippel, 1961; Eagland et al., 1974).

A method is presented here for estimating the number-average molecular weight of junction zones and their standard free energy of formation from measurements of the apparent shear modulus of very weak gels at concentrations of polymer just above the gel threshold.

THEORY

THE THEORY of rubber elasticity (Treloar, 1975) gives the following relationship between shear modulus (G) and the weight concentration of polymer (c):

$$G = cRT/M_c \qquad (1)$$

where M_c is the number average molecular weight of polymer chains joining adjacent cross-links (active chains), R is the gas constant and T the absolute temperature.

Consider unit volume of solution. If M is the number average molecular weight of the polymer, the number of polymer molecules in this unit volume is $N_{av}c/M$, where N_{av} is Avogadro's number. Thus if there are J junction zones per unit volume, the number of junction zones per polymer molecule is $JM/N_{av}c$ and the number of active chains per polymer molecule is

$$\{JM/N_{av}c - 1\}$$

If "free ends" of the polymer chains are neglected and M_J is the number average molecular weight of the junction zones, it then follows that the number average molecular weight of the active chains is

$$M_c = \left\{M - \frac{M_J M J}{N_{av}c}\right\}\left\{\frac{JM}{N_{av}c} - 1\right\}^{-1} \qquad (2)$$

Defining $[J] = J/N_{av}$ (i.e. the "molar concentration" of junction zones) and substituting Eq. (2) in Eq. (1) then gives the expression for the shear modulus:

$$G = \frac{RTc}{M} \cdot \frac{M[J] - c}{M_J[J] - c} \qquad (3)$$

The quantity [J] can be calculated from the law of mass action if it is assumed that the cross-linking loci can act as independent species in solution (Pouradier, 1967; Eldridge and Ferry, 1954). Thus,

$$[J] = K_J[L]^n \qquad (4)$$

where K_J is the association constant, [L] the "molar concentration" of crosslinking loci and n the average number of these that associate to form a junction zone. From Eq. (4) and mass balance it then follows that

$$K_J = [J]M_J^n \{n(c - M_J[J]\}^{-n} \qquad (5)$$

Eq. (3) and (5) combined define the relationship between shear modulus and concentration. There are four unknown quantities (M, M_J, K_J and n) and given sufficient sets of values of G and c it is possible to optimize their values to fit experimental data.

It is important to emphasize that the applicability of this treatment is severely restricted by the assumptions that have to be made. Firstly, the theory of rubber elasticity cannot validly be applied unless the polymer chains retain sufficient flexibility for the gel network to obey Gaussian statistics. Mitchell and Blanshard (1979) have considered this problem in connection with polysaccharide gels and concluded that chains of more than 1000 monomer units would be required. The chain lengths of the common gelling polysaccharides, such as pectin, carrageenan, and alginate, are of this order of length (Glicksman, 1969) but in their gels the average length of the active chains must be substantially shorter except in very dilute solutions close to the gel threshold. Protein gels, particularly gelatin, appear to follow the theory of rubber elasticity to higher concentrations – presumably because of the greater flexibility of polypeptide chains compared with polysaccharides (Mitchell, 1976). The second major assumption is that the formation of junction zones can be treated as an equilibrium process, subject to the law of mass action [Eq. (4)]. There is an obvious objection to this in that gels are very slow to equilibrate, displaying hysteresis in their properties (Rees, 1969). This is a consequence of the high degree of cooperativity in the formation of junction zones; the process is, in some respects, analogous to the folding and unfolding (denaturation) of proteins in which many individually weak interactions cooperatively maintain the native structure. Protein

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Table 1—Number average molecular weights of the polymer (M) and characteristics of the junction zones (M_J, K_J and n) calculated for gelatin and pectin gels

	Concentration range (g/L)	Gel pcint (g/L)	M	M_J	K_J	n
Gelatin	11.7 – 19.8	10	50 000	13 400	4.1×10^5 [a]	3.18
Pectin: DM = 64.9	2.03 – 4.06	1.83	88 000	6 230	70[b]	1.97
DM = 69.7	1.51 – 2.87	0.72	113 000	10 600	420[b]	2.03
DM = 72.3	0.66 – 3.29	0.56	249 000	25 000	1380[b]	208
DM = 93.2	2.4 – 4.0	1.46	92 000	92 000	–	201

[a] In units of L^2mol^{-2} [b] In units of $Lmol^{-1}$

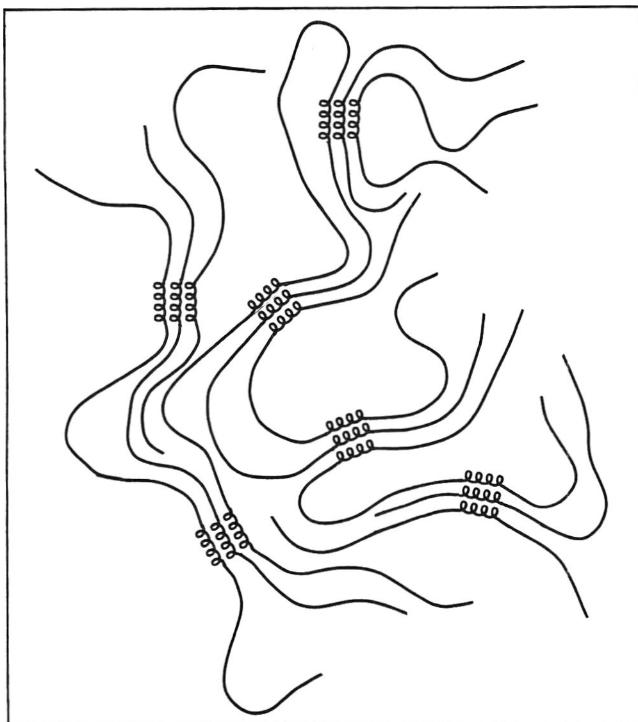

Fig. 1—Schematic diagram of the gelatin gel network. The junction zones are sections of collagen triple helix in which lengths from three polymer chains are intertwined.

denaturation can be reversible and can be discussed in terms of equilibrium thermodynamics if the solutions are sufficiently dilute to avoid protein-protein interactions and subsequent aggregation of partly refolded protein molecules (Tanford, 1970). The melting of crystalline polymers can also be treated as a reversible process (Mendellkern, 1964). Thus it seems reasonable to consider the formation and dissociation of junction zones as an equilibrium process, particularly in dilute gels with relatively few junction zones.

MATERIALS & METHODS

GELS were prepared from pectins of different chain length and different degrees of esterification as described in the accompanying paper (Oakenfull and Scott, 1984). All but one of the pectins were purified commercial products characterized by standard techniques (McCready, 1970). The other was a highly esterified product produced by treating one of the commercial pectins with diazomethane (Hough and Theobald, 1963).

Measurements of shear modulus were made in a thermostatted room (25 ± 1°C) using the method of Saunders and Ward (1954).

Calculations

The experimental data required are values of shear modulus measured at concentrations close to the gel threshold. The calculations can be carried out in the following sequence.

(1) Select trial values for M, M_J and n. It should usually be possible to estimate values within an order of magnitude by using reasonable expectations for these parameters.

(2) Estimate, by extrapolation, the concentration (c_0) at which the shear modulus becomes zero. From Eq. (3), it follows that at this concentration, $[J] = c_0/M$ and K_J can be calculated from Eq. (5). This procedure reduces the number of adjustable parameters from four to three, vastly simplifying the calculations.

(3) Using Eq. (3) and (5), calculate G for each concentration for which experimental values of G are available.

(4) Calculate the sum of squares of the differences between the experimental and calculated values of G.

(5) Adjust M, M_J and n so as to minimize the sum of squares of differences. There are many methods for finding the minimum in the sum of squares rapidly and with high precision (see, for example, Kowalik and Osborne, 1968). For this work, with smoothly varying continuous functions it is not necessary to go past direct search methods. All that is required is to calculate the sums of squares with different values for the parameters to determine the changes needed to reduce the sums of squares sequentially until the minimum is found.

RESULTS & DISCUSSION

MEASUREMENTS of shear modulus at concentrations close to the gel point have been reported for gelatin (Saunders and Ward, 1954) and full details of the results obtained for pectin gels are reported in the accompanying paper (Oakenfull and Scott, 1984). The values of M, M_J and n (and consequently K_J) which gave the best fit to these experimental data were calculated and the results are summarized in Table 1.

In the case of gelatin the molecular weight was within the range usually found for commercial gelatin preparations (Harrington and von Hippel, 1961; Veis, 1964). Particularly interesting is the finding that the best value for the number of cross-linking loci per junction zone (n) is close to 3. There is strong evidence that the junction zones in gelatin gels have the collagen triple helix structure (Vies, 1964) but it has been suggested that these helical regions might be partly intramolecular (Eagland et al., 1974). The evidence presented here suggests that the triple helices are in fact wholly intermolecular. The molecular weight of the junction zones (M_J) indicates that the average number of amino acid residues in the triple helix is 142, or 47 per strand of protein. This represents about 16 turns of the triple helix and about 5 turns of the collagen super-helix (Rich and Crick, 1961). Thermodynamic arguments lead to qualitatively the same conclusion indicating 8–33 turns of collagen triple helix per junction zone (Rees, 1969; Pouradier, 1967).

The molecular weights calculated for the four pectins are again within the range usually reported for commercial preparations (Jordan and Brant, 1978). For these gels the results indicate that there are two cross-linking loci per junction zone which is in accordance with other evidence (Rees and Wight, 1971). The size and thermodyanmic stability of the junction zones are proportional to the square of the fraction of esterified glucuronic acid groups of the pectin. This result appears to be correct since the square of the degree of esterification is proportional to the probability of esterified groups being opposed in adjacent segments of

—Continued on page 1110

Increase in Emulsification Activity of Soy Lecithin-Soy Protein Complex by Ethanol and Heat Treatments

MOTOHIKO HIROTSUKA, HITOSHI TANIGUCHI, HIROSHI NARITA, and MAKOTO KITO

ABSTRACT

Soy proteins were associated with soy lecithin. Soy protein isolate (SPI), 7S and 11S proteins complexed with lecithin were similarly affected by ethanol treatment to increase emulsification activity. By this procedure, the conformation of these proteins was changed resulting in aggregation. Though emulsification activity of the lecithin SPI complex was apparently increased by boiling for 1 min, this treatment only caused the 11S protein component to aggregate resulting in better emulsification activity, regardless of whether it was complexed with lecithin or not. The increase in emulsification activity caused by ethanol treatment was not affected by NaCl, whereas the increase by heat treatment was reduced by NaCl. It seems likely that ethanol and heat treatments change the conformation of soy proteins in different ways.

INTRODUCTION

THERE ARE MANY FOODS containing oil together with water. It is necessary for the taste and texture of these foods to make oil and water stable emulsions. Emulsifying activity is one of the most important functional properties of food proteins. Separate hydrophobic and hydrophilic regions are distributed in protein molecules (amphipathic structure). This structure is required for the formation of emulsions. Soy protein has good functional properties for food processing (Kinsella, 1979). Fortification of emulsifying activity of soy protein increases the utilizability of soy protein in various foods.

Many approaches have been carried out to change the emulsification properties of soy protein. Partial hydrolysis (Aoki et al., 1980; Aoki and Matsuura, 1976), ethanol treatment (Aoki et al., 1981), and acylation (Aoki et al., 1978) of soy protein improve the emulsion stability under acidic conditions. These modifications may increase the hydrophobicity of soy protein. Recently, fatty acids were covalently incorporated into food proteins by Haque and Kito (1982), Haque et al. (1982), and Haque and Kito (1983a, b). The lipophilized proteins have high emulsification activity.

Commercial soy lecithin is a well-known natural emulsifier for foods. This is produced as a by-product of soybean oil refining. To increase the use of soy lecithin would be important from the view point of by-product utilization (Szuhai, 1983). Kito and coworkers have shown that lecithin has a strong affinity for soy proteins (Ohtsuru et al., 1976, 1979; Kanamoto et al., 1977; Ohtsuru and Kito, 1983).

In this paper, we attempt to develop a soy lecithin-soy protein complex in food systems by enhancing its emulsifying activity.

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MATERIALS & METHODS

Materials

Defatted soy flour (moisture 5%, oil content 0.3%, crude protein 50%, nitrogen solubility index 90) was obtained from Fuji Oil Co. (Osaka, Japan). Powdered commercial lecithin (SLP white) was obtained from True Lecithin Industrial Co. (Mie, Japan).

Preparation of 7S and 11S proteins and soy protein isolate (SPI)

Crude 7S and 11S protein fractions were prepared by Thanh's method (Thanh et al., 1975). The water soluble fraction was extracted from defatted soy flour at 50°C for 1 hr with a 15-fold excess of 63 mM Tris-HCl buffer pH 7.8 containing 10 mM 2-mercaptoethanol and 0.025% NaN_3 (Buffer 1). The insoluble residue was removed from the slurry, and the supernatant was adjusted to pH 6.6 with 5N HCl. The supernatant was then dialyzed at 1°C for 3 hr against 63 mM Tris-HCl buffer, pH 6.6, containing 10 mM 2-mercaptoethanol and 0.025% NaN_3. After the dialysis, the precipitate was collected by centrifugation at 3000 x *g* for 10 min. The precipitate was used as crude 11S protein fraction. The supernatant which was separated from 11S protein fraction was adjusted to pH 4.6 with 5N HCl and centrifuged at 3000 x *g* for 10 min. The precipitate was used as the crude 7S fraction. Soy protein isolate (SPI) was obtained as follows: The water soluble fraction was extracted as described above. Then, the pH was adjusted to 4.6 and centrifuged at 3000 x *g* for 10 min. The precipitate was used as SPI fraction. All protein fractions (11S, 7S, and SPI) were resolubilized in Buffer 1 and dialyzed to the same buffer overnight before use. The protein concentration of each fraction was adjusted to 4% in the same buffer.

Soy lecithin-soy protein complex

A suspension of commercial soy lecithin in water was added to 4% soy protein solution and sonicated using a Branson sonifier (Type-200, 150W) to form the complex. The dry weight ratio of protein/lecithin was usually adjusted to 4:1.

Ethanol treatment

An equal volume of 99% ethanol was added to a lecithin-protein complex solution while stirring. After standing for 30 min at 25°C, the pH of the mixture was adjusted to 4.6 with HCl and the solution was centrifuged at 3000 x *g* for 10 min. The precipitate was washed twice with 20-fold distilled water to remove excess residual ethanol and then resolubilized in Buffer 1. Lecithin-protein complex thus treated has been termed "ethanol treated lecithin-protein complex." Protein solution without lecithin was also treated in a similar manner to obtain the "ethanol treated protein."

Heat treatment

Soy protein solution (2%) was heated in boiling water for 1 min and the temperature achieved in the protein solution was 95°C. Then, the solution was cooled immediately in water at 4°C. Protein solution without lecithin which was thus treated has been termed "heat treated protein" and the complex has been termed "heated lecithin-protein complex."

Determination of protein

Protein was determined by the colorimetric method of Bradford (1976) using a Bio-Rad protein assay kit. Lyophilized SPI was used as a standard protein.

Emulsification activity (EA)

The turbimetric and point determination method of Pearce and Kinsella (1981) was used for our purpose. Soybean oil was added to 3 mL of 0.4% protein solution containing 50 mM Tris-HCl buffer pH 7.5. The amount of soybean oil was varied in the range of 0.2, 0.5, 1.0, 2.0, and 3.0 mL. The mixture was emulsified for 1 min by sonication. Ten μL of the emulsion were mixed with 20 mL of 1% sodium dodecyl sulfate and the absorbance at 600 nm was measured. The data were expressed as relative values (percentage) compared to the untreated control samples. Duplicate experiments were carried out, and highly reproducible data were obtained.

Determination of hardness of emulsions

Solutions of the ethanol-treated and untreated lecithin-SPI complex and SPI were powdered using a spray drier. Temperature of inlet and outlet air flow was 160° and 80°C, respectively. Moisture of the samples after drying was controlled at 6 - 8%. The spray dried sample (20g) and soybean oil (90g) were added to 100g water and mixed with a homogenizer at 12000 rpm for 5 min. The resulting emulsionwas then placed in a tube (diameter; 50 mm, height; 10 mm) and hardness determined as viscosity with a texturometer (Zenken GTX-2). The diameter for the plunger was 10 mm and voltage was 10V. The values of hardness were expressed by mm/V.

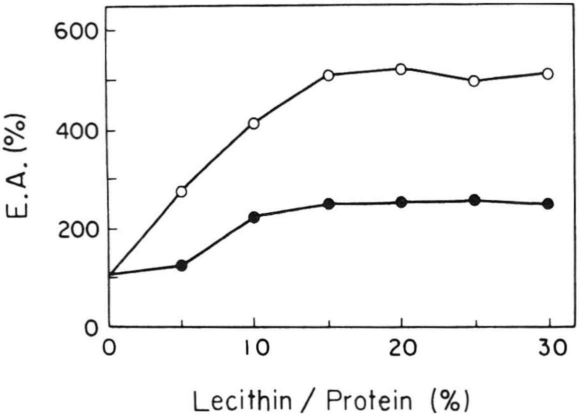

Fig. 1—Relationship between the lecithin to soy protein isolate ratio and emulsification activity following ethanol treatment. With (○) and without (●) ethanol treatment. EA – emulsification activity.

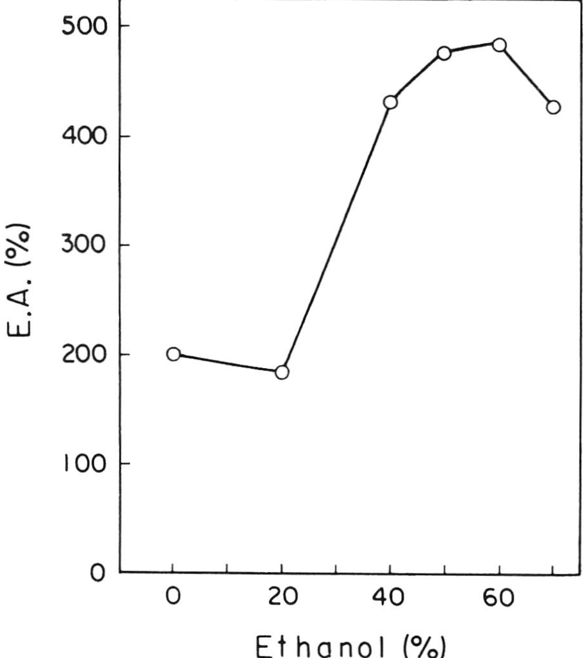

Fig. 2—Effect of ethanol concentration on theincrease of emulsification activity of the lecithin-soy protein isolate complex. EA – emulsification activity.

Gel filtration

Protein solution was dialyzed against 35 mM phosphate buffer pH 7.5 containing 10 mM 2-mercaptoethanol, 0.4M NaCl, and 0.025% NaN_3. The sample was then applied on the Sepharose CL-4B column (1.0 x 85 cm) equilibratec with the same buffer. The amount of applied protein was 10 mg, and the flow rate was 20 mL/hr. After fractionation, absorbance (280 nm) of each fraction was meausred.

Circular dichroism

Circular dichroism spectra between 200 and 250 nm were recorded by a JASCO model J-500 (Japan Spectroscopic Co., Tokyo, Japan). The concentration of protein was 2 mg/mL and the conditions of measurement were at 20°C, pH 7.8, at a scanning cpeed of 20 nm/min. The data were converted to mean residual ellipticity [θ] by using a data processor (JASCO model DP-5000): Difference spectra of the treated and untreated samples were obtained using the ame process.

RESULTS

Ethanol treatment of soy lecithin-soy protein complex

As the ratio of soy lecithin to soy protein isolate (SPI) increased, emulsification activity (EA) of the protein increased (Fig. 1). When the lecithin-SPI complex was treated with 50% ethanol, EA of the complex increased five times as much as that of SPI. However, the EA of the untreated complex was 2.5 times as much as that of SPI. Effect of lecithin on EA reached a plateau when its ratio to SPI was 15-20%.

The ethanol treatment at a concentration of 40-60% at 25°C for 30 min was most effective in increasing the EA of the lecithin-protein (1:4) complex (Fig. 2). Under the above conditions, EA of 7S and 11S proteins and that of SPI were examined. EA of ethanol treated 7S, 11S, and SPI was similar to those of the respective control proteins (Fig. 3). Though 7S, 11S, and SPI showed a twofold increase in their EA following the formation of complex with lecithin as compared with the control proteins, ethanol treatment enhanced the EA of the lecithin-protein complex almost fivefold.

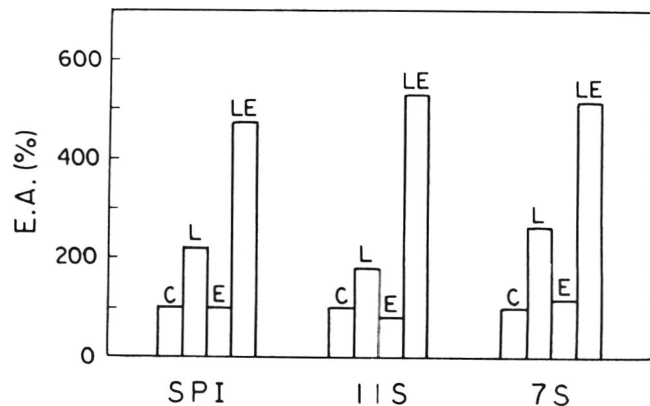

Fig. 3—Effect of ethanol treatment on emulsification activities of soy protein isolate (SPI), 11S, and 7S proteins. C, control; L, lecithin protein complex; E, ethanol treated protein; LE, ethanol treated lecithin-protein complex; EA – emulsification activity.

The hardness (viscosity) of emulsion prepared from the spray-dried proteins, soybean oil and water (20:90:100, W/W/W) was measured with a texturometer. The emulsion containing the ethanol treated lecithin-SPI complex was a creamy paste and showed 4.1 mm/V, whereas those of the lecithin-SPI complex, ethanol treated SPI, and SPI gave 2.8, 2.0, and 2.0 mm/V, respectively.

Circular dichroism (CD) and gel chromatography of the ethanol treated lecithin-protein complex

In order to examine the conformational changes and molecular sizes of the lecithin-protein complexes following ethanol treatment, CD and gel filtration experiments were carried out. The CD spectrum of the ethanol treated lecithin-11S protein complex is shown in Fig. 4A. In the region of 210–230 nm, the spectrum differed the untreated complex. The difference spectrum suggested the conformational changes were caused by the ethanol treatment (Fig. 4B). Similar changes were also seen in the case of the lecithin-7S protein complex following ethanol treatment (Fig. 5A and B).

The untreated lecithin-protein complex of 11S or 7S protein showed gel filtration profiles that were similar to those of the original protein (Fig. 6 and 7). However, the ethanol treatment caused aggregation of the proteins. EA of the main peak (fractions 1–4) shown in Fig. 6 and 7 retained the original activities of the respective samples applied on the column (Table 1). The elevated EA was found in the aggregated fraction (fraction 1). This indicated that lecithin and proteins formed a stable complex which could not be separated by gel filtration as described previously (Ohtsuru et al., 1976; 1979; Kanamoto et al., 1977; Ohtsuru and Kito, 1983).

Heat treatment of soy lecithin-soy protein complex

It has been reported that heat treatment caused the polymerization of soy protein (Mori et al., 1981). We examined the effect of heat treatment. When a 4% solution of the lecithin-SPI (1:4) complex was heated in boiling water, EA of the complex increased about twice that of SPI (Fig. 8). The EA of the lecithin-SPI complex was apparently increased four times as much as following heat treatment, compared to the unheated SPI. The maximum increase of EA by the heat treatment was lower than that observed with the ethanol treatment. Enhancement of the EA by heating was different from that by the ethanol treatment (Fig. 9). Heat treatment equally increased EA of 11S and the lecithin-11S protein complex. In other words, this change was caused regardless of whether the lecithin-protein complex was formed or not. The EA of 7S protein was increased by the formation of lecithin 7S protein complex. However, the EA of the lecithin-7S protein complex was not increased by heat treatment.

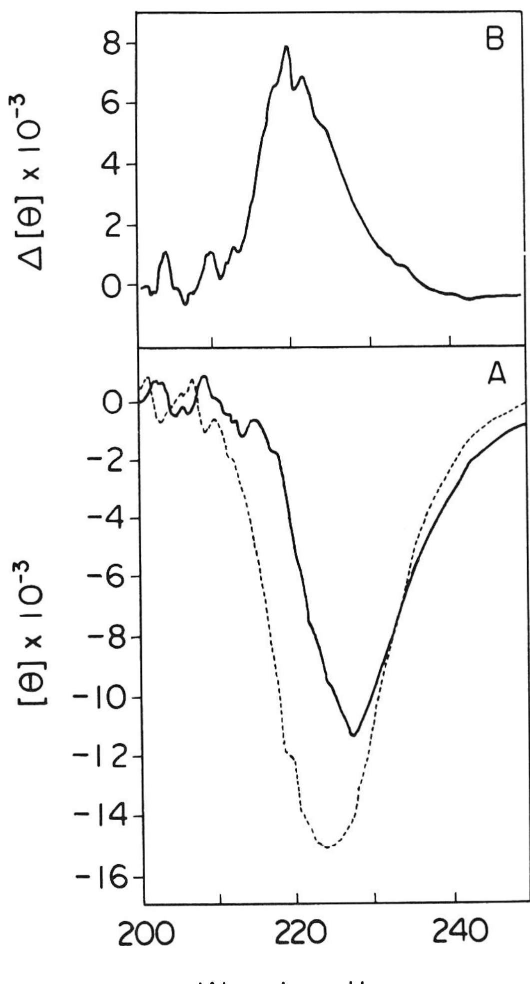

Fig. 4—CD spectra of lecithin-11S protein complex: (A) ethanol treated lecithin-11S protein complex (dotted), lecithin-11S protein complex (solid). (B) difference spectrum of A.

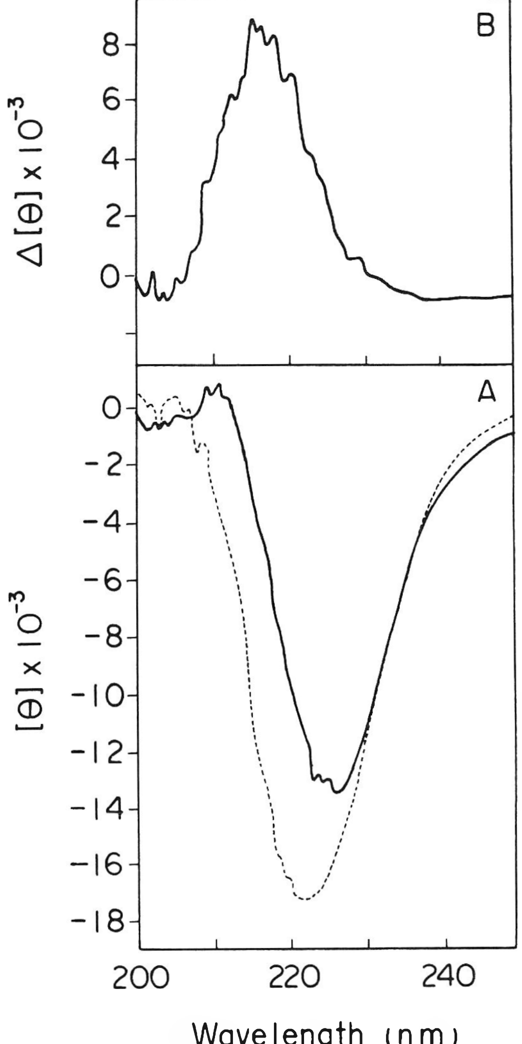

Fig. 5—CD spectra of lecithin-7S protein complex: (A) ethanol treated lecithin-7S complex (dotted), lecithin-7S protein complex (solid). (B) difference spectrum of A.

CD and gel chromatography of the heat treated soy lecithin-soy protein complex

Difference CD spectrum of the heat treated lecithin-7S protein complex, compared with the untreated sample, indicated little conformational changes caused by heat (Fig. 10A), in contrast to a large change in conformation of the heated lecithin-11S protein (Fig. 10B). Such different effects of heat on the conformation of 7S and 11S proteins may support the differences in the change of EA (Fig. 9). However, aggregation of both 7S and 11S proteins was caused by heat treatment (Fig. 11) as was the case following ethanol treatment.

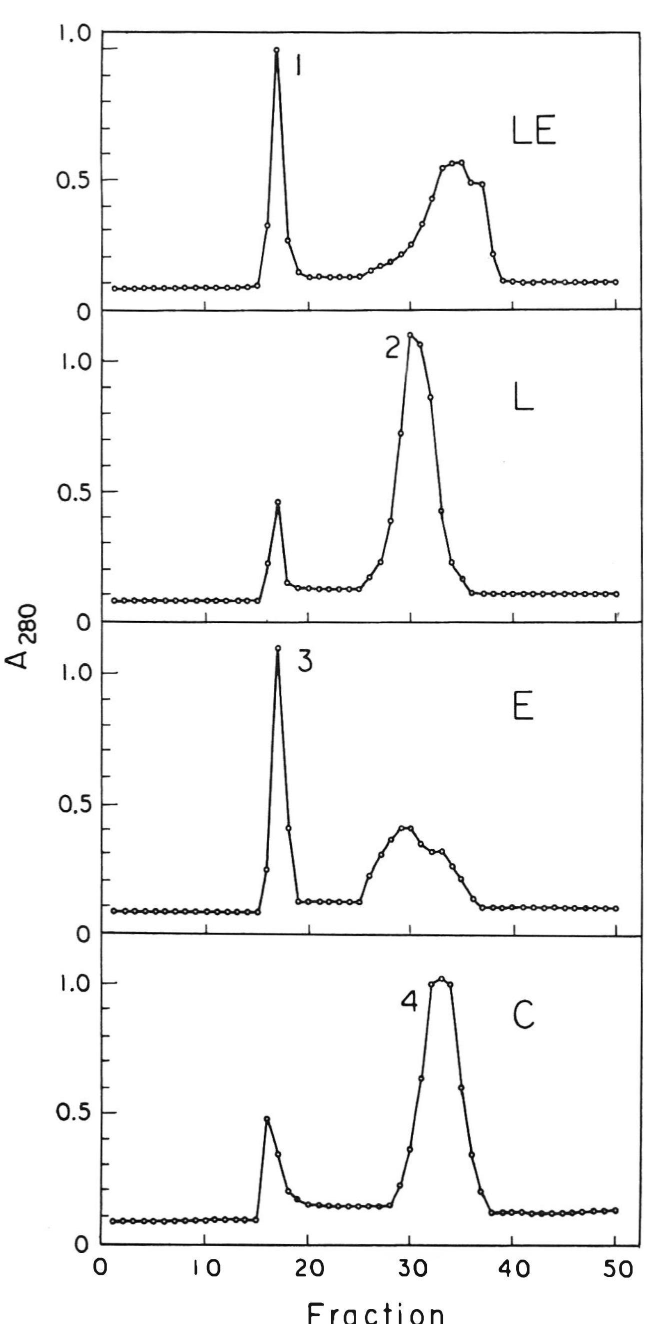

Fig. 6—Gel filtration of 11S protein. C, 11S protein; E, ethanol treated 11S protein; L, lecithin-11s protein complex; LE, ethanol treated lecithin-11S protein complex. Peaks 1-4 were used for the determination of emulsification activity.

Effect of NaCl on increase of EA caused by ethanol and heat treatments

In order to see whether the increase in EA following ethanol and heat treatments was caused by the same mechanism, effect of NaCl concentration was examined. The increase in EA by ethanol treatment was independent of NaCl concentration, whereas enhanced effect of heat was reduced as NaCl concentration increased (Fig. 12).

DISCUSSION

WE HAVE previously shown that soy proteins form a stable

Table 1—Emulsification activity of fractions separated by gel filtration[a]

Protein	Fraction 1	Fraction 2	Fraction 3	Fraction 4
7S	450	145	130	100
11S	330	100	105	100

[a] Fraction numbers as shown in Fig. 6 and 7.

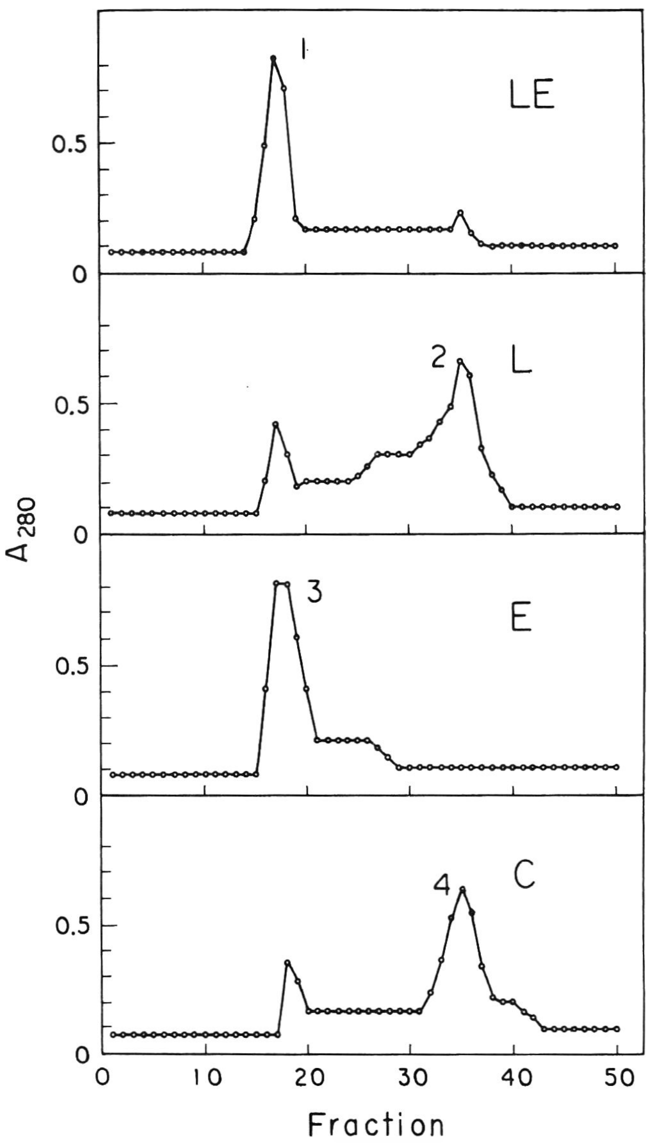

Fig. 7—Gel filtration of 7S protein. C, 7S protein; E, ethanol treated 7S protein; L, lecithin-7S protein complex; LE, ethanol treated lecithin-7S protein complex. Peaks 1-4 were used for the determination of emulsification activity.

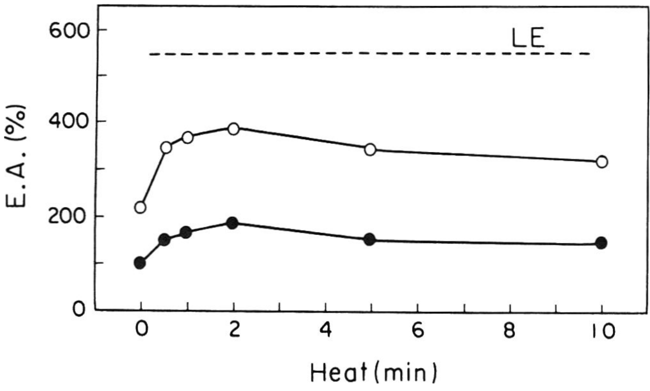

Fig. 8—Effect of heat treatment on emulsification activities of soy protein isolate (SPI) and lecithin-SPI complex. ●, SPI; ○, lecithin-SPI complex; LE, ethanol treated lecithin-SPI complex.

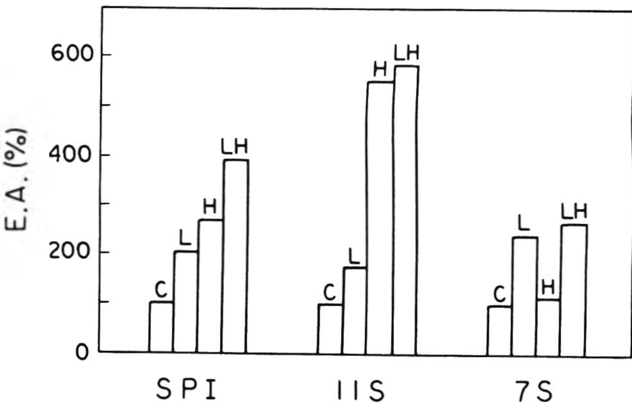

Fig. 9—Effect of heat treatment on emulsification activity of soy protein isolate (SPI), 7S, and 11S proteins. C, control; L, lecithin-protein complex; H, heat treated protein; LH, heat treated lecithin-protein complex; E.A., emulsification activity.

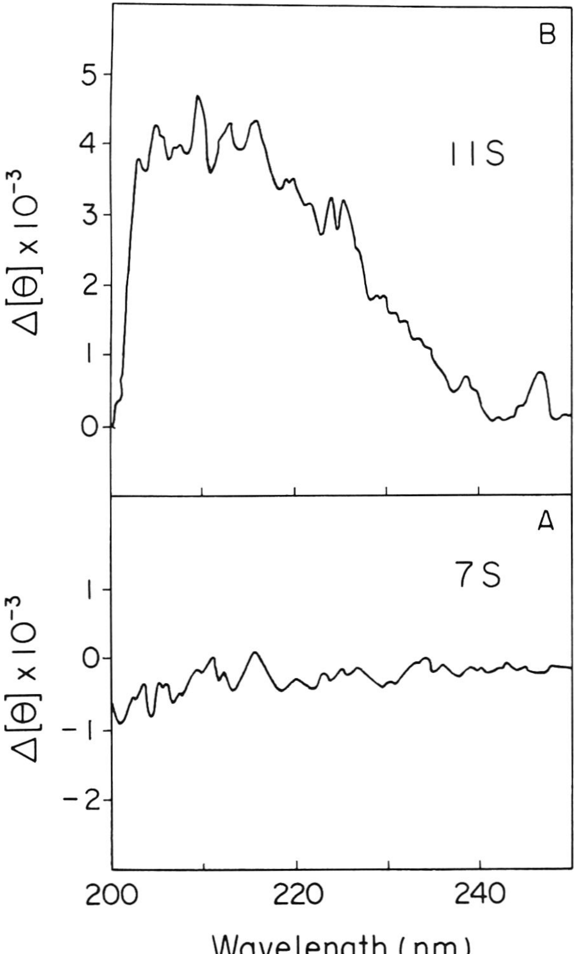

Fig. 10—Difference CD spectra of heat treated lecithin-7S (A) or 11S (B) protein complex as compared to respective untreated complexes.

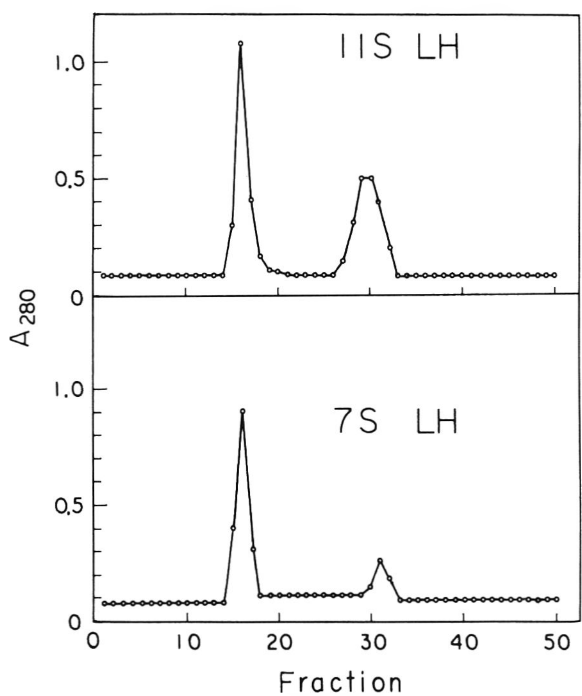

Fig. 11—Gel filtration of heated lecithin-7S or 11S protein complexes. 7S LH, heat treated lecithin-7S protein complex; 11S LH, heat treated lecithin-11S protein complex.

complex with lecithin by sonication (Ohtsuru et al., 1976, 1979; Kanamoto et al., 1977; Ohtsuru and Kito, 1983). The lecithin soy-protein complex has a higher EA than does soy protein. The EA of the lecithin-soy protein complex was enhanced by ethanol treatment. The conformation of soy proteins was changed by this treatment, and their aggregation occurred. In the process of aggregation, lecithin was firmly associated with the soy protein, and the final products of partially denatured lecithin-soy protein complex may contain polymerized proteins with amphipathic structure where hydrophobic surface may have increased.

Heat treatment, however, affected only 11S protein to be aggregated to increase EA, regardless of whether lecithin-protein complexes were formed or not, whereas aggregation of 7S protein caused by heat treatment did not increase its EA. Heat denaturation of 11S protein was suppressed by high ionic strength (Iwabuchi and Shibasaki, 1981). Reduction of enhancement of EA in the presence of high NaCl concentration (Fig. 12) suggests relationship between denaturation and the increas of EA. However, increase of EA by ethanol treatment was not affected by NaCl. Hence, aggregation of soy proteins seems necessary but not sufficient for enhancement of EA.

From some lines of evidence, it seems likely that ethanol and heat treatments change the conformation of soy

protein in different ways. It is noted that undesirable flavors were removed during the ethanol treatment. The ethanol treated lecithin-SPI complex is expected to be widely used for human foods.

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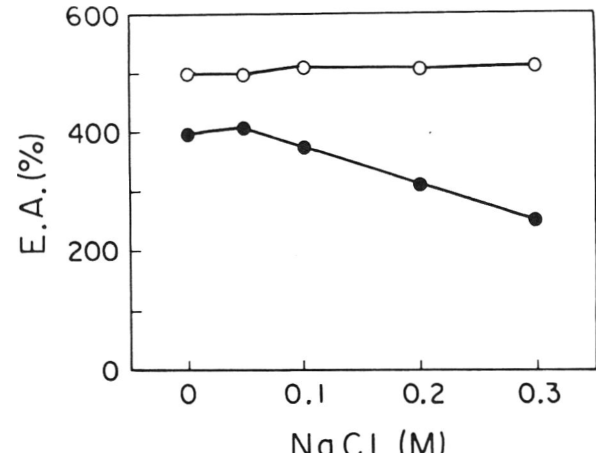

Fig. 12—Effect of NaCl on increase in the emulsification activity of ethanol or heat treated lecithin-soy protein isolate (SPI) complex. ○, ethanol treated lecithin-SPI complex; ●, heat treated lecithin-SPI complex.

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JUNCTION ZONES IN NONCOVALENT GELS . . . From page 1104

polymer. Such ester groups in close proximity would contribute hydrophobic stabilization to the junction zones (Oakenfull and Scott, 1984).

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Calcium Fortification of Soy Milk with Calcium-Lecithin Liposome System

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ABSTRACT

Calcium ion was enveloped with a membrane system before addition to soy protein to prevent soy protein from being coagulated and precipitated by calcium ion. Soy lecithin was first sonicated in calcium salt solution to envelop the calcium ion with a liposomal structure composed of lecithin. Then, the calcium-lecithin liposomes were added to soy protein solution. Precipitation and coagulation were not observed in this soy protein-lecithin liposome system containing 60 mM Ca^{2+}. By this method, it was possible to prepare calcium fortified soy milk containing more calcium (120 mg/100g) than in cow's milk. These results suggest that this calcium-lecithin liposome system is useful for calcium fortification of soy milk.

INTRODUCTION

MUCH ATTENTION has been given to soy milk and soy protein beverages as they are considered to be healthy foods. Soybean contains high quality protein, tocopherol and essential fatty acids. In addition, soy milk has no cholesterol (Hashimoto, 1983; Saio, 1983). However, soy milk is inferior to cow's milk in two areas. One is a problem of off-flavor (so called beany flavor). Different procedures have been used to diminish this flavor (Fujimaki et al., 1968; Chiba et al., 1979). The other is the problem of poor mineral balance for human nutrition, which has not yet been solved.

Calcium content in soybeans is about 240 mg/100g (O'Dell and Boland, 1976; Rham and Jost, 1979). It is difficult to increase the calcium concentration in soy milk, since soy protein is coagulated and precipitated by calcium-protein interaction (Saio et al., 1967). The coagulated soy protein is not suitable for beverages. Weingartner et al. (1983) made a soy protein beverage containing a heterogenous suspension consisting of calcium citrate and calcium phosphate. However, they found it difficult to make a homogenous and stable soy protein solution containing more than 100 mg/ 100g free calcium ion.

Kito and coworkers (Ohtsuru et al., 1976, 1979; Ohtsuru and Kito, 1983; Kanamoto et al., 1977) showed that soy lecithin formed a multi-layer membrane structure by sonication and that the lecithin was associated with soy proteins.

The purpose of this study was to prepare soy milk containing a high amount of calcium. In order to prevent soy protein from being coagulated by calcium ion, we tried to envelop calcium ion within a membrane system to prevent direct contact of calcium ion with soy protein.

MATERIALS & METHODS

Materials

Dehulled soybean and defatted soy flour were obtained from Fuji Company (Osaka, Japan). Powdered soy lecithin was obtained from True Lecithin Industrial Company (Mie, Japan). All other reagents used in this study were of food additive grade according to Japanese regulations.

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Preparation of lecithin liposomes

One part of a 10% lecithin in water suspension was added to 19 parts of aqueous solutions of calcium lactate of various concentrations. The mixtures were then sonicated using a Branson sonifer (Type 200, 150W) for 10 min at full power to make the mixture clear. The pH of every solution was adjusted to 7.0 with 1N HCl or 1N NaOH before sonication.

Preparation of soy protein isolate (SPI)

Twelve parts of hot water (50°C) were added to one part of the defatted soy flour, and the pH was adjusted to 7.5 with 5N NaOH. The mixture was stirred for 1 hr at 50°C, then centrifuged at 3000 × *g* for 10 min. The supernatant was adjusted to pH 4.5 with 1N HCl and centrifuged at 3000 × *g* for 10 min. The resulting precipitate was washed with a tenfold volume of water and centrifuged again at 3000 × *g* for 10 min. Finally, the precipitate was dissolved in an appropriate amount of water by being neutralized with 5N NaOH. This was used as the SPI solution.

Preparation of natural soy milk

Hot water (95°C) was added (8.7-fold w/w) to soaked dehulled soybean (10A soybeans from U.S.A.) and ground with a food mixer. The slurry was centrifuged at 3000 × *g* for 10 min and the supernatant was used as natural soy milk.

Stability

The stability of soy protein-lecithin complex solution containing calcium salt was determined at 4°C for 1 wk. The sample which had no precipitation and coagulation after 1 wk was considered as stable.

Determination of protein

Protein content was determined by the colorimetric method of Bradford (1976) using a Bio-Rad protein assay kit. Lyophilized SPI was used as a standard protein.

RESULTS & DISCUSSION

Calcium-soy lecithin liposomes

When lecithin suspension was sonicated with salt solution, lecithin formed liposomes in which salts were entrapped (Bangham et al., 1974). However, some of the cations may locate around negatively charged polar head groups of phospholipids, and/or exist as unencapsulated state. Commercial soy lecithin contains more phosphatidylethanolamine than egg yolk lecithin (Hasegawa, 1981) and it is aggregated by calcium ion. This aggregation suppresses the formation of commercial soy lecithin liposomes. Table 1 shows the minimum concentrations of calcium salts which cause aggregation and precipitation of soy lecithin. For these experiments, 0.5% (w/w) soy lecithin was used. The minimum concentrations that caused lecithin to be aggregated were different among these salts. Calcium chloride had the highest ability to precipitate soy lecithin. Calcium citrate, calcium gluconate, and calcium carbonate did not seem appropriate for calcium fortification because of their low solubility. Calcium lactate, which had less aggregation effect on soy lecithin compared with the other salts, was considered as the most suitable compound for

Table 1—Minimum concentration for aggregation of lecithin by calcium salts

Calcium salt	Minimum conc (mM)
Calcium chloride	7
Calcium lactate	16
Calcium D-pantothenate	10

Table 2—Minimum concentration of chelating agents for preventing soy lecithin (0.5%) from being aggregated by 1M calcium chloride

Chelating agent	Minimum conc (M)
EDTA-4Na	1.0-1.5
Sodium citrate	1.5-2.0
Sodium meta-phosphate	2.0-2.5
Sodium poly-phosphate	3.5-4.0

Table 3—Effect of sodium citrate on soy lecithin aggregation by 30 mM calcium lactate[a]

Sodium ictrate/ Calcium lactate (M/M)	0	0.25	0.5	0.75	1.0	1.5	2.0
Aggregation	X	X	X	●	●	○	○

[a] The concentrations of Ca^{2+} and lecithin were 30 mM and 0.5%, respectively. X, aggregation; ●, turbid; ○, clear solution.

Table 4—Effect of lecithin on soy protein isolate (SPI) coagulation by a mixture of calcium lactate and sodium citrate[a]

Lecithin (%)	0	0.125	0.25	0.5	0.75	1	2
Coagulation	X	X	●	○	○	○	○

[a] The concentrations of reagents are as follows: calcium lactate, 30 mM; sodium citrate, 45 mM; soy protein isolate (SPI), 3.5%. X, coagulation; ●, turbid; ○, clear solution.

Table 5—Effect of calcium contents on coagulation of soy protein isolate (SPI)[a]

Ca^{2+} (mM) / (mg/100g)	0 / 0	10 / 40	20 / 80	30 / 120	40 / 160	50 / 200	60 / 240
No heat −soybean oil +SPI	○	○	○	○	○	○	○
Heat −soybean oil +SPI	○	○	●	X	X	X	X
Heat +soybean oil +SPI	○	○	○	○	●	X	X

[a] The concentrations of reagents are as follows: lecithin, 0.5%; soybean oil, 3%; SPI, 3.5%. Ca was added in a mixture of calcium lactate and sodium citrate (1:1.5). The heating was carried out at 37°C for 10 min. Symbols are the same as in Table 4.

calcium fortification of soy milk. Calcium lactate, however, aggregated soy lecithin when it was used at high concentration. In order to reduce the soy lecithin-calcium interaction, some chelating agents were used. Various amounts of chelating agents were added to 1M calcium ions derived from calcium chloride, and chelating effects (suppresion of aggregation) were examined (Table 2). Ethylenediamine tetraacetic acid (EDTA-4Na) was most effective. This reagent is permitted as a food additive but is not popular in Japan. Sodium citrate was therefore employed as a chelating agent because of its acceptability and food safety. Table 3 shows the effect of sodium citrate on lecithin aggregation caused by 30 mM calcium lactate. More than 30 mM sodium citrate decreased the soy lecithin aggregation.

Interaction of SPI with calcium-soy lecithin liposome system

In order to prepare the lecithin liposomes with calcium lactate-sodium citrate (1:1.5) complex, 60 mM calcium lactate and 90 mM sodium citrate were sonicated with various lecithin suspensions. The mixture was then added to an equal volume of 7% SPI solution, and the stability of the solution was examined (Table 4). The lecithin concentration was varied in the suspension that was sonicated. Presence of 30 mM calcium lactate and 45 mM sodium citrate coagulated SPI with or without unsonicated lecithin. However, at and above 0.5% lecithin, the calcium-protein interaction was completely suppressed.

The coagulating effect on SPI of various concentrations of calcium salt with lecithin liposomes was examined. The concentrations of calcium were varied from 0 - 60 mM with 0.5% soy lecithin, and the molar ratio of calcium lactate/sodium citrate was maintained at 1:1.5. Then the effect of heating and soybean oil on this system were examined (Table 5). The SPI solution was stable up to a calcium content of 60 mM without heating. However, when heated at 100°C for 10 min, the SPI was coagulated by calcium ions at and above 20 mM. Since SPI was not coagulated at 100°C for 10 min, this may probably be due to the liberation of free calcium ions surrounding the polar head groups of soy lecithin or uncappsulated calcium ions. On the other hand, the system which was supplemented with soybean oil was stable even up to 40 mM Ca^{2+}. The system without SPI was stable when heated.

Preparation of calcium fortified model soy milk

Calcium fortified soy milk which contained more calcium ion (120 mg/100g) than cow's milk (100 mg/100g) was prepared by the use of soy lecithin liposome system as mentioned above. All reagents were divided into two groups (groups A and B). Group A consisted of calcium lactate (920 mg), sodium citrate (1.3g), soy lecithin (500 mg), and water (40.8g) with sonication. Group B consisted of soybean oil (3g), SPI (3.5g), and water (50g). The samples were prepared by three different processes (T-1, T-2, and T-3). T-1 was prepared by mixing groups A and B directly and storing at 4°C. T-2 was treated by the same process as T-1 except for sterilization (100°C, 10 min) before storage at 4°C. In T-3, groups A and B were sterilized separately at 100°C for 10 min, immediately cooled to 4°C, then mixed and stored at 4°C. After storage for 1 wk, a little precipitation was observed in T-2 sample but there was no precipitation nor coagulation in T-1 and T-3 samples.

Calcium fortified natural soy milk

Calcium fortified natural soy milk was prepared by employing the liposome system. Twenty milliliters of lecithin liposome solution containing 130 mM calcium lactate, 195 mM sodium citrate and 2% lecithin was sterilized and added to 67 mL of natural soy milk and stored at 4°C for 1 wk. During storage, the soy milk was very stable without any signs of precipitation and coagulation.

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Phytic Acid Content of Crude, Degummed and Retail Soybean Oils and Its Effect on Stability

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ABSTRACT

An anion exchange method was employed to determine phytate content of industrial crude and degummed soybean oil and soybean oil purchased at retail. The phytate content of crude oil ranged from 48.9-339.4 ppm and degummed oil ranged from 3.9-50.9 ppm phytate. Degumming removed from 16.1-97.1% of the phytate. Retail oil did not contain detectable amounts of phytate. Peroxide values were measured to test the effect of added phytate on the stability of oil exposed to heat, light and copper. In these tests phytate did not affect oil stability.

INTRODUCTION

PHYTIC ACID has been reported present in soybeans and in many soy products (Thompson and Erdman, 1982). A great deal of interest has been generated by phytate, the hexaphosphate salt of inositol, due to its suggested responsibility for lowered mineral bioavailability. Phytate has not been previously reported as a component of crude, degummed or retail soybean oil.

Previous work in this laboratory has been to identify and quantitate phospholipids using high performance-thin layer chromatography. HPTLC plates of polar lipid fractions of both crude and degummed oil contain significant amounts of phosphorus at the origin (Racicot and Handel, 1980), and as phytate remains at the origin under similar conditions, the presence of phytate is implicated. Al-Kahtani et al. (1984) reported that the addition of $FeCl_2$ aided in the removal of phosphorus during degumming perhaps by precipitation of phytate. Phytate is known to bind with metal ions such as ferric, a prooxidant of oil (Beal and Lancaster, 1951). Therefore, the presence of phytate in oil may be advantageous in limiting the autoxidation of soybean oil. Phytate combined with lecithin has been patented as an antioxidant in Japan (Tsukagoshi et al., 1972).

Sodium phytate is a concentrated source of phosphorus, containing 28.2% phosphorus by weight, while phospholipids are 3-5% phosphorus and 6-7% in the lyso forms (Chapman, 1980). All sources of phosphorus in crude oil are important since the reduction in phosphorus in degummed oil is used to measure degumming efficiency and because phosphorus content is one of the criteria used to determine the quality of refined oil (Beal et al., 1956).

Trace amounts of protein have been reported in crude soybean oil (Kito et al., 1979) and were concluded to be in a reverse micellar structure. Since phytate has been demonstrated to bind readily with protein (Prattley and Stanley, 1982), this may explain why hydrophilic phytate is present in nonpolar soybean oil. The purpose of this study was to quantitate phytic acid in crude, degummed and retail soybean oils and determine the effect of phytate on oil stability when added to retail soybean oil.

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MATERIALS & METHODS

Samples and storage

Crude and degummed soybean oils were collected on four consecutive days from four different soybean oil processing plants in Lincoln, NE, Decatur, IL, Fort Wayne, IN, and Stuttgart, AR. Samples were collected from one of the processors at two times during the year (November and February). The samples, which were previously analyzed for total phosphorus and phospholipid content (Racicot and Handel, 1983), were stored below 0°C and were thawed, and warmed until clear, before being analyzed for phytate. Retail soybean oil was purchased from a local store.

Ion exchange method

Analysis of phytate was attempted by a modification of the method of Harland and Oberleas (1977). Oil (5 mL) was homogenized with 100 mL 0.3N HCl, followed by separation and analysis of the aqueous extract, but recovery of phytate was not quantitative. The following method was then developed. One gram of anion exchange resin (AG1-X8, Bio-Rad Laboratories) was packed into a 7 mm i.d. x 150 mm Econo-column (Bio-Rad Laboratories) with 5 mL 1N HCl in chloroform:methanol:water (60:40:1, v/v/v). Excess Cl^- was eluted from the column with 30 ml chloroform:methanol: water (60:30:1, v/v/v). The elutant is Cl^- free when a precipitate no longer forms with 1M $AgNO_3$. The sample (0.2g) was dissolved in 25 mL chloroform:methanol:water (60:30:1, v/v/v) and applied to the column. Nonphyate phosphorus compounds were eluted with 15 mL 0.2N HCl in chloroform:methanol:water (60:30:1, v/v/v). Phytate was then eluted with 30 mL 1.5N aqueous HCl and collected in 30 mL micro-Kjeldahl flasks.

Phosphorus determination

Solvent was removed from phytate containing eluates by evaporation over a micro burner. The remaining contents were digested and analyzed for phosphorus according to the method of Rouser et al. (1966) using half amounts of reagents. Total phosphorus was determined by the procedure of Racicot and Handel (1983).

Analysis of standard phytate

Sodium phytate (BDH, Gallard-Schlesinger) for use as a standard was dried at 110°C for 2 hr to determine moisture content. Gradient elution of phytate from the ion exchange column was performed according to the method of Bartlett (1982). The gradient, from 0-1N HCl, was used to determine the degree of phosphorylation of the BDH phytate. Aliquots of a standard solution of sodium phytate (50 μg/mL) were added to oil samples, then dissolved in 25 mL chloroform:methanol:water (60:30:1, v/v/v) and applied to the ion exchange column in order to determine phytate recovery.

Stability of oil with phytate

Phytate was added to commercial soybean oil as an aqueous solution (35 mg/mL), mixed with the oil using a Polytron homogenizer (Brinkmann Instruments) then dried in a vacuum oven. In a similar manner, $CuSO_4$ was added to samples. Phytate was added to produce phytate concentrations of 42 and 128 ppm and Cu^{+2} to a 10^{-5}M concentration. Oil samples were placed in 120 mL glass jars (60 mm o.d. x 70 mm) and capped, then stored at rest in an incubator at 63 ± 2°C. For the light stability test, phytate was added at 50 μg/g and 150 μg/g levels with Cu^{+2} at 10^{-5}M. These samples were exposed to a 40W cool white fluorescent light at a distance of 100 mm. Oxidative stability was determined on the sample aliquots by the peroxide value (PV) as determined by the AOCS Official Method Cd 8-53 (AOCS, 1973). PV was measured biweekly and plotted as a function of time exposed to heat or light. Linear regres-

Table 1—Phytate content and percent of total phosphorus of crude and degummed oil

Company	Day	Crude		Degummed		% Phytate removed by degumming
		Phytate (ppm)[a]	% of Total P	Phytate (ppm)[a]	% of Total P	
A-1	1	78.6 ± 1.3	3.2	18.5 ± 0.4	7.4	76.5
	2	72.3 ± 4.5	3.6	43.9 ± 3.1	46.5	39.8
	3	78.0 ± 0.3	3.1	19.3 ± 10.6	10.7	75.3
	4	85.2 ± 9.3	3.6	31.1 ± 1.9	24.1	57.0
A-2	1	77.8 ± 0.3	3.5	36.5 ± 2.6	19.8	53.1
	2	55.4 ± 8.3	2.4	46.5 ± 6.0	30.2	16.1
	3	79.0 ± 11.0	3.5	19.2 ± 3.9	2.4	75.7
	4	48.9 ± 8.9	2.2	38.8 ± 6.5	7.5	20.7
B	1	145.2 ± 0.1	4.1	6.4 ± 1.8	7.6	97.1
	2	133.9 ± 14.1	4.1	3.9 ± 1.5	6.0	94.6
	3	126.2 ± 17.6	3.7	6.8 ± 2.0	13.6	95.6
	4	123.1 ± 5.4	3.8	4.3 ± 1.0	9.6	96.5
C	1	120.1 ± 0.4	3.8	25.8 ± 0.2	8.5	72.8
	2	142.8 ± 0.7	5.0	50.9 ± 10.7	9.9	81.9
	3	187.0 ± 9.1	6.6	25.2 ± 2.9	6.3	83.9
	4[b]	156.8 ± 2.8	4.5	—	—	—
D	1	175.5 ± 2.3	5.7	32.3 ± 3.8	6.7	86.3
	2	217.1 ± 7.5	7.4	29.7 ± 5.2	7.5	81.6
	3	339.4 ± 23.0	12.6	39.9 ± 4.2	10.6	88.2
	4	190.4 ± 7.7	7.2	20.9 ± 2.2	6.1	89.0

[a] Average of duplicate determinations.
[b] Degummed sample was broken in shipment.

sion was used to determine slopes of the lines and analysis of variance was used to determine differences between slopes at the 0.05 level of significance.

RESULTS & DISCUSSION

Recovery of standard phytate

Gradient elution produced a single peak of phosphorus for the standard phytate insuring that all six inositol carbons were phosphorylated. Standard solutions containing 20, 40 and 50 μg phytate were applied to ion exchange columns and 94.5, 97.2 and 94.4% of the phytate was recovered, respectively. A second trial produced yields of 103.7-109.7% from duplicate standards containing 20, 30 and 40 μg phytate.

Phytate content

The phytate content of crude soybean oil samples ranged from 48.9-339.4 ppm (Table 1). Phytate in degummed oil ranged from 3.9-50.9 ppm; thus, percentages of phytate removed by degumming ranged from 16.1-97.1%. Oils from company A showed the most variation in the amount of phytate removed and on the average less phytate was removed by company A during degumming than the other companies. Company B was the most efficient in removing phytate (94.6-97.1%). Companies C and D removed 72.8-89.0% of phytate. Retail oil did not contain detectable phytate using this method of analysis; therefore, phytate must be removed in the processing steps subsequent to degumming.

Effect of phytate on stability

Peroxide values of oils containing added phytate and exposed to heat or light were not significantly different from controls held under the same conditions. In addition, oils containing both phytate and copper and oils containing just copper were also not significantly different from the controls. This unexpected finding from samples containing copper means the effects of phytate as an antioxidant were not exhibited in this test system. It may be necessary to test the effects of these hydrophilic materials in an emulsion to see their effect.

In this study we have shown that phytate was responsible for a small but measurable portion of the phosphorus of crude soybean oil. In degummed oil, phytate accounted for a larger percentage of phosphorus present in the oil and in one case almost half of the phosphorus came from phytate. The amount of phytate removed by degumming varied depending on the company. Phytate was not shown to have any effect on oil stability in the system tested.

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Color Codes for Paired Preference and Hedonic Testing

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ABSTRACT

The use of color as a product coding device for consumer sensory testing was studied. Simple paired preference and hedonic tests were conducted using eight brightly colored, 2-inch round labels on a carbonated lemon-lime beverage packaged in 12-oz aluminum cans. We concluded that little or no bias exists for the eight colors when used in paired preference tests. For hedonic testing, an order/color bias was noted for some pairs of colors. With appropriate order balancing those effects can be understood and reduced. Therefore, color coding is a valid technique that can be used for consumer sensory testing.

INTRODUCTION

AN IDEAL CODE for sensory tests is one that is random, easily identified, quick and easy to apply, easily recalled, and one that has no bias associated with it. It is important that the subject's response reflects the product and not the code. The code must not give any clues to the identity of the product (Schaefer, 1979).

Some coding procedures do not meet those criteria. For example, two-digit codes and single-letter or two-letter codes are avoided because they may represent more than one meaning (for example, a panelist's initials or age) (Schaefer, 1979).

Although little research has been done on color coding of products, there has been some research pertaining to the use of color for architectural design codes and target coding. Evans et al. (1980) concluded that in the study of interior designs of buildings, people with the benefit of color coding performed better on way-finding tasks, were more accurate at locating places in the building with a surveyors transit, and made fewer errors in recall and recognition of the building's floor plan than people without the benefit of color codes.

Christ (1975) reported that a subject's search time for targets could be at least 43% less with the use of color codes rather than alphanumeric symbols. Jones (1962) also concluded that color has a particular value in coding applications with visual search tasks and suggested that the real virtue of color as a coding device is in category coding (i.e. partially redundant coding) with search tasks.

The "colors" black and white have been studied by Color Meaning Tests and Preschool Racial Attitude Measures by numerous authors with the conclusion that white is viewed more positively than black by young children (Best et al., 1976, 1975; Boswell and Williams, 1975; Iwawaki et al., 1978; Williams et al., 1975; Williams and Stabler, 1973). Those results indicate that color can produce a "biasing" effect.

For sensory testing, Gould et al. (1957) apparently used color codes successfully but did not present data comparing the codes. A three-digit code has been used with success and is recommended (Schaefer, 1979) as nonbiasing. However, the research by Evans et al. (1980) and Christ (1975) implies sample selection would be faster and more accurate with colors than with other codes. In addition, color codes were selected for study because they are neat, easily obtained, and require no additional time spent in printing or typing codes on the labels. Our experiences with color codes indicate that panelists could select the appropriate samples quickly with few errors – an advantage that is important in unsupervised testing situations, such as home use testing.

The purpose of this study was to examine the use of color as a non-biasing method of coding sensory samples. Both preference and hedonic tests were conducted.

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MATERIALS & METHODS

Materials

Round labels (Avery International Corporation – Avery Label Division), 2 inches in diameter, were purchased in eight glossy finished colors (Table 1). The labels (codes), one color per can, were applied to a carbonated lemon-lime flavored beverage packaged in plain 12-oz aluminum cans. The labels were backed with a solid black coating (Avery-Blackout P4) which did not allow the silver color of the can to show through and distort the color of the label. The beverage was a single formula produced in one batch to eliminate differences in products throughout the studies.

Consumers

The panelists represented consumers in the St. Louis metropolitan area who were members of various community groups (church groups, scout troops, etc.). Three hundred thirty-six consumers participated in the scaling test (45% male, 55% female) and 2,352 consumers participated in the preference test (39% male, 60% female, 1% unidentified). The youngest participant was 12 and the oldest was over 60 years of age. No one age group dominated the studies. The participants in the hedonic test were not used for the preference test.

Methods

The tests were self-administered in the home without direct supervision. For both the hedonic tests and paired preference tests, each person received two cans of the same product labelled with two different colors. The judges were asked to chill the products, to taste the products in a specific sequence, and either to give hedonic ratings to each of the two coded samples or to select the preferred sample.

For the hedonic test panelists used 9-point scales anchored at the ends with "like extremely" and "dislike extremely". There were 28 combinations and two order presentations (56 pair combinations), replicated six times. For the paired preference test the judges

Table 1—Description of colors and hedonic means for beverages with varying color codes

Avery #	Color description	Mean[a]
F-05	Rubine Red	5.9
F-12	Moly Orange	5.8
F-21	Medium Chrome Yellow	5.8
F-50	PTA Green	6.2
F-62	Cyan (Process Blue)	6.1
F-94	Purple (Rhodamine B)	5.5
F-Br1	Brown	6.3
F-K1	Black	5.8

[a] Hedonic Scale: 1—dislike extremely to 9—like extremely

determined a preference between the two coded products using a forced-choice paired preference test. Again there were 56 pair combinations with each combination replicated 42 times.

Analysis of data

The hedonic data were subjected to analysis of variance and t-test to determine if order of testing was significant. Additional analyses were run with combined data.

The preference data were subjected to a paired comparison test and chi-square analysis to determine order effects.

RESULTS & DISCUSSION

Hedonic test

The hedonic test resulted in no significant differences among colors (p=0.76) and no significant order effect (t-value=0.98). Hedonic means were generated for each color (Table 1) irrespective of order because order had no effect. The values for the product labelled with specific colors ranged from 5.5–6.3. Those means were within the range expected from the experimental variation of this population. We do not believe that the lack of an order effect eliminates the need to balance order because other characteristics of the test or products (such as carry-over of flavor) may influence ratings. However, the lack of an order effect for color coding bias in hedonic testing reduces the number of parameters and, thus, the complexity of the balancing that must be controlled.

Table 2—Beverage preference results for orders of pairs and total data for color codes

		Preference P-Value			
A Color	B Color	Received A color first	Received B color first	Combined results	Color x Order Chi-Square P-Value
Yellow	Red	0.64	0.44	0.741	0.28
Yellow	Blue	0.64	0.16	0.101	0.51
Yellow	Green	0.28	0.88	0.230	0.51
Yellow	Orange	0.88	0.64	0.447	0.83
Yellow	Brown	0.88	1.00	0.741	0.83
Yellow	Black	0.09+	0.64	0.327	0.08+
Yellow	Purple	0.88	0.16	0.156	0.38
Red	Blue	0.88	0.16	0.156	0.38
Red	Green	0.64	0.28	0.156	0.66
Red	Orange	0.88	0.44	0.582	0.38
Red	Brown	0.88	1.00	0.741	0.83
Red	Black	0.44	0.02*	0.012*	0.26
Red	Purple	1.00	0.64	0.582	0.66
Blue	Green	0.88	0.88	0.582	1.00
Blue	Orange	0.09+	1.00	0.156	0.19
Blue	Brown	0.88	0.88	1.000	0.66
Blue	Black	0.64	0.44	0.741	0.28
Blue	Purple	0.44	0.88	0.582	0.38
Green	Orange	0.02*	0.44	0.230	0.02*
Green	Brown	0.64	0.44	0.038*	0.26
Green	Black	0.88	0.88	1.000	0.66
Green	Purple	0.88	0.28	0.230	0.51
Orange	Brown	0.28	0.16	0.741	0.05*
Orange	Black	0.28	0.64	0.582	0.19
Orange	Purple	0.28	0.88	0.447	0.27
Brown	Black	0.64	0.88	0.447	0.83
Brown	Purple	0.16	0.44	0.582	0.08+
Black	Purple	0.28	0.88	0.101	0.27

+ ≤p 0.10
* ≤p 0.05

Preference test

The preference test indicated little bias contributed by color combinations (Table 2). There were no significant differences in product preference for most pairs of color codes. For the total data (28 pairs irrespective of order of presentation) only the red/black and green/brown pairs resulted in a significant preference for either color in the pairs. For the individual combinations (order considered), the combinations green first/orange second and black first/red second were significant (p=0.02) for the first color in each pair. The combinations yellow first/black second and blue first/orange second were also significant (p=0.09) for the first color of the pairs.

Chi-square analysis (Table 2), indicated that the interaction of color and order was important, and resulted in significance for: black/yellow (p=0.08), orange/green (p=0.02), brown/orange (p=0.05), and brown/purple (p=0.08). Those results indicated that the limited color-coding bias of preference samples that does occur may be order related. Thus, the data reinforces the well-known and common practice of balancing order of presentation during preference testing.

The finding of significance for preference results and not for hedonic testing is not inconsistent. The products in a pair were exactly the same, differing only in the color code. For the preference test panelists might resort to external cues (such as the color code) in order to give a forced preference response to products that tasted alike. In the hedonic test panelists could (and some did) give exactly the same score to the products. External cues probably would have less effect.

CONCLUSIONS

WE BELIEVE, based on the hedonic results and the overall preference results, that the colors having a significant outcome were influenced either by order (which is easily balanced) or were random occurrences. On the basis of this study, color coding of samples does not seem to produce a pronounced bias and, thus, is effective for sensory testing with consumers. Further testing, especially on different product and package types, is needed to confirm those results.

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Emulsifying Properties of an N-Terminal Peptide Obtained from the Peptic Hydrolyzate of αs1-Casein

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ABSTRACT

A peptide corresponding to the 23 N-terminal residues of αs1-casein, αs1(1-23), was purified from a peptic hydrolyzate of αs1-casein. The emulsifying activity of αs1(1-23) varied depending on the concentration. When the concentration was higher than 2%, αs1(1-23) showed a similar emulsifying activity to αs1-casein in neutral pHs. The emulsion capacity of αs1(1-23) was about one half that of αs1-casein. The extent of adsorption of αs1(1-23) onto the oil globule surface was markedly high. In the acidic pH region (pH 5.5 - 3.0), αs1(1-23) maintained a high emulsifying activity, whereas αs1-casein lost its emulsifying activity. These results suggest that αs1(1-23) acts as an emulsifying agent and probably can be used in some foods such as processed cheese or fermented milk products.

INTRODUCTION

THE PEPTIDES, which have smaller molecular sizes and simpler structures than proteins, can be expected to have different functional properties from those of proteins. The functional peptides may be useful in various food processing operations, but very little information has so far been available on the functional properties of peptides.

Studies on the functional properties of peptides are also important to elucidate the functionality of proteins. It is considered that the functional properties of a protein are affected by many structural factors, e.g., the disposition of amino acids, molecular size, shape, conformation, net charge, hydrophobicity, and protein-protein interactions (Horiuchi and Fukushima, 1978; Kinsella, 1981). Because of the complexity of a protein structure, the relationship between the structure and functionality of proteins remains obscure. Information given for peptides having simpler structures will contribute to a better understanding of these aspects.

Caseins, the major milk proteins, have good functional properties and are widely used in the manufacture of dairy and nondairy foods. Since the primary structures of caseins have already been established (Mercier et al., 1971; Ribadeau-dumas et al., 1972; Jolles et al., 1972. Mercier et al, 1973), studies using caseins (or their peptides) may provide good models to elucidate the protein (peptide) structure-functionality relationships.

The purpose of the present study was to isolate a functional peptide from αs1-casein and investigate its properties, especially for emulsification.

MATERIALS & METHODS

Preparation of αs1-casein B

Crude αs1-casein was prepared from fresh raw skim milk according to the method of Zittle et al. (1959). The purification of αs1-casein was performed on a DEAE-Sephacel column (3.5 x 20 cm). The elution of αs1-casein from the column was carried out with 0.02M phosphate buffer (pH 6.5) containing 4M urea under a linear gradient of NaCl (0.1 - 0.6M). The αs1-casein fraction obtained here showed a single band on polyacrylamide slab gel electrophoresis.

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Partial proteolysis of αs1-casein

Limited cleavage of the αs1-casein was carried out by using pepsin, chymosin or plasmin as follows. Pepsin digestion: To a 2% αs1-casein solution (w/w) in 0.1M Na-acetate buffer (pH 6.4) was added a solution of crystalline pepsin (Sigma, from porcine stomach) to give an enzyme/substrate ratio of 1/330 (w/w) and the mixture was then incubated at 30°C. Chymosin digestion: To a 2% αs1-casein solution (w/w) in 0.1M Na-acetate buffer (pH 6.4) was added chymosin (Difco) to give an enzyme/substrate ratio of 1/50 (w/w) and the mixture was incubated at 30°C. Plasmin digestion: To a 2% αs1-casein solution (w/w) in 5 mM phosphate buffer (pH 7.0) was added plasmin (Sigma, from porcine plasma) to give an enzyme/substrate ratio of 1/5000 (w/w) and the mixture was incubated at 37°C.

Isolation of small peptides

A 2% solution of αs1-casein in 0.1M Na-acetate buffer (pH 6.4) was incubated with pepsin at 30°C for 30 min (E/S ratio, 1:330, w/w). After incubation, the solution was adjusted to pH 8.0 and allowed to stand for 20 min to inactivate the enzyme. The solution was acidified to pH 4.6 with 0.1N HCl and centrifuged at 1840 x *g* for 10 min. The supernatant (peptide fraction) was repeatedly diluted and concentrated on a G-01T membrane filter (ULVAC Service Corp.) at 4°C until free from salts. The solution was then freeze-dried. The precipitate was solubilized at pH 7. desalted by dialysis against water at 4°C and freeze-dried.

Polyacrylamide gel electrophoresis

Slab gel electrophoresis was performed according to the method of O'Farrell (1975) using 7.5% acrylamide gel containing 4.5M urea. Urea-sodium dodecyl sulfate-polyacrylamide gel electrophoresis (urea-SDS-PAGE) was by the method of Swank and Munkres (1974) using 12.5% acrylamide gel (bis:acrylamide ratio = 1.15 (w/w)). Staining was performed with coomassie blue R-250 for the protein.

High performance liquid chromatography (HPLC)

A high performance liquid chromatograph (Jasco Trirotor SR-2) was fitted with a Finepak-sil ODS column (4.6 x 250 mm) and a UV detector at a wavelength of 230 nm. One hundred micrograms of the sample were injected and eluted with 0.1% trifluoroacetic acid (TFA)-acetonitrile as a mobile phase at a flow rate of 1.0 ml/min. The concentration of the mobile phase modifier (acetonitrile) was increased linearly from 0 - 80% over 60 min (1.33%/min).

Amino acid analysis

A major peptide separated on HPLC was evaporated to remove the TFA and acetonitrile, and was dissolved in 6N HCl. The solution was degassed and hydrolyzed at 110°C for 24 hr. The hydrolyzate was analyzed with an automatic amino acid analyzer (Hitachi Model 835).

Emulsifying properties

Emulsifying activity was evaluated according to the procedure described by Pearce and Kinsella (1978) with slight modification. An emulsion was prepared by homogenizing a 2% (w/w) protein solution and 20% (w/w) soybean oil (Sanko Pharmaceutical Co., Japan) at 30°C with a Polytron PTA-7 (Kinematica, Switzerland) for 3 min at full speed (19,500 rpm). The emulsion was immediately diluted with 0.1% SDS and its turbidity was measured at 500 nm. Emulsifying activity was presented in terms of Emulsifying Activity Index (EAI).

Emulsion capacity was measured according to Webb et al. (1970) using a 0.5% protein solution and soybean oil at room temperature. The rate of oil addition was 0.20 - 0.25 mL/sec and the mixing was done with a Polytron PT-20 at 19,500 rpm. The transition point was detected by electrical resistance measurement. Emulsion capacity (EC) was expressed in ml of oil/mg of protein.

Protein adsorption onto the oil globule surface

The emulsion prepared with 2% protein and 20% soybean oil was centrifuged at 11,000 x *g* for 30 min. An aliquot was taken from the aqueous portion and the protein content was measured by the procedure of Lowry et al. (1951). The extent of protein adsorption was expressed as:

$$\frac{\text{Total protein content} - \text{protein content in the aqueous phase}}{\text{Total protein content}} \times 100$$

RESULTS

Emulsifying properties of αsl-casein digested by proteases

No marked increase in the EAI was observed by plasmin or chymosin digestion of the αsl-casein. However, the EAI of the αsl-casein was increased by pepsin-hydrolysis (Fig. 1). It was assumed that some peptides having good emulsifying activities were produced in the pepsin digests, and isolation of the peptides was attempted.

Analysis of the small peptide fraction

A small peptide fraction was obtained from the pepsin hydrolyzate of αsl-casein by the procedure described in Materials & Methods. The yield of the peptide fraction was about 80 mg/g αsl-casein. The PAGE patterns of αsl-casein, the peptide fraction, and the pH 4.6-precipitated fraction are shown in Fig. 2. The peptide fraction showed no band on slab-PAGE but gave a single band (molecular weight approx 3000 daltons) on urea-SDS-PAGE.

The result of HPLC analysis of the peptide fraction on an ODS-column is shown in Fig. 3. Only one major peak was observed, indicating the homogeneity of this fraction. The eluate corresponding to this peak was collected and its amino acid composition was analyzed.

The amino acid composition of this fraction closely resembled that of the 1-23 residues of αsl-casein B as shown in Table 1. We concluded that this peptide corresponded to the N-terminal 1-23 residues of αsl-casein [αsl(1-23)].

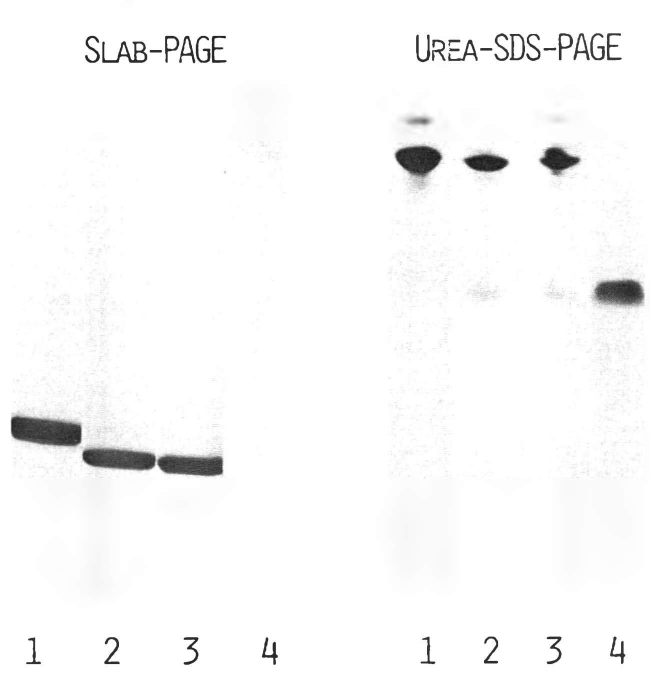

Fig. 2—Electrophoretic patterns of αsl-casein and αsl-casein digests: (1) αsl-casein; (2) pepsin-digest; (3) pH-4.6-ppt of pepsin-digest; (4) pH 4.6-sup of pepsin-digest (small peptide).

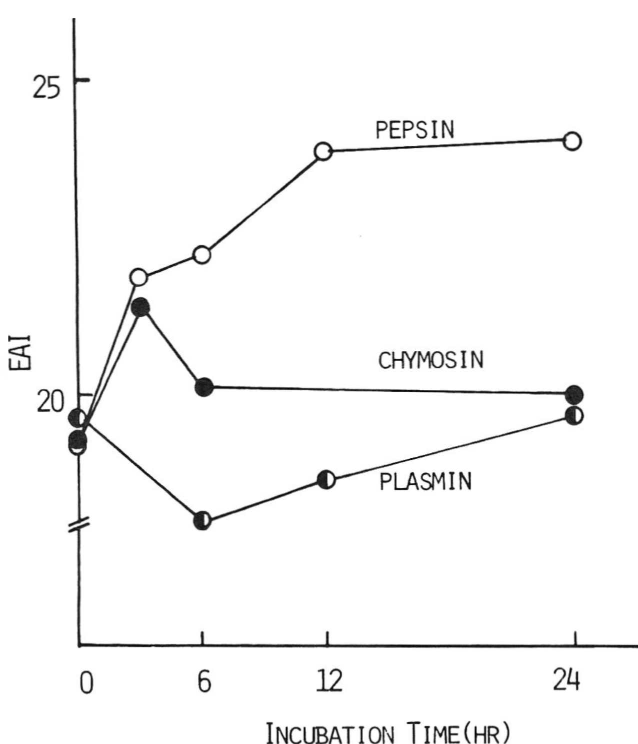

Fig. 1—Effect of protease digestion on the EAI of αsl-casein. Each point is the average of three observations.

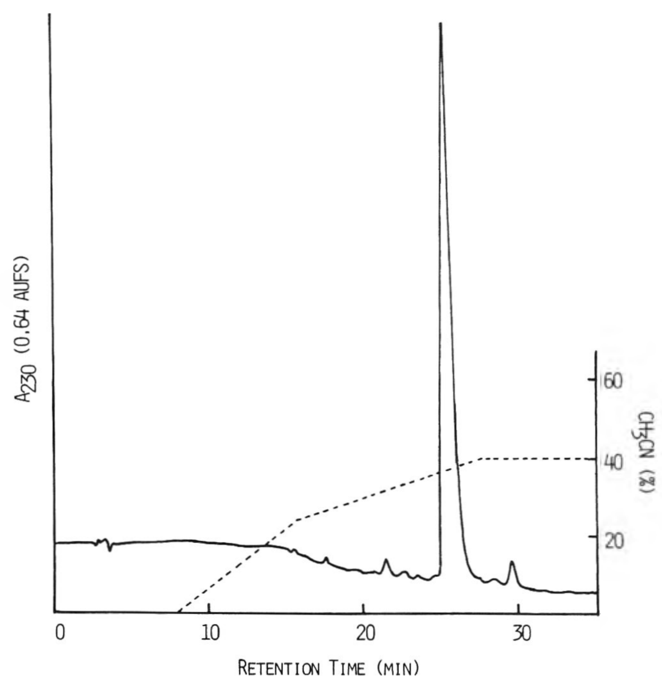

Fig. 3—HPLC patterns of the αsl-casein peptide fraction on Finepak-sil ODS (4.6 x 250 mm i.d.). Primary solvent: 0.1% TFA; secondary solvent: acetonitrile. The gradient profile is shown by the dotted line.

Emulsifying properties of αsl(1-23) peptide

Three parameters – EAI, EC, and the extent of protein adsorption – were determined to evaluate the emulsifying properties of αsl(1-23) peptide, comparing with αsl-casein and the pH 4.6-precipitated fraction [the main component is αsl(24-199)].

As shown in Table 2, the EAI of αsl(1-23) was similar to that of αsl-casein when a 2% protein (peptide) solution was used for the measurement, while the EC of αsl(1-23) was remarkably low, compared with that of αsl-casein. The EAI of αsl(1-23) was shown to be dependent on the concentration used. Fig. 4 shows that the EAI of αsl(1-23) decreased remarkably when the concentration was lower than 2%, whereas that of αsl-casein was not concentration dependent. The adsorption of αsl (1-23) onto an oil globule was extremely high. In the case of a 2% solution, more than 70% of this peptide associated with the oil globule surface on emulsification.

The emulsifying activities of this peptide and αsl-casein were measured as a function of pH. The results are shown in Fig. 5. The emulsifying activity of αsl-casein was a minimum at around pH 5 because of isoelectric precipitation, while αsl(1-23) maintained its high emulsifying activity in the acidic pH region (pH 5.5 – pH 3.0).

Table 1—Amino acid composition of a peptide obtained from the peptic hydrolyzates of αsl-casein

Amino acid	Molar ratio	Nearest integer	αsl(1-23)
Asx	2.17	2	2
Thr	0.25	0	0
Ser	0.22	0	0
Glx	3.82	4	4
Pro	3.00	3	3
Gly	1.27	1	1
Ala	–	–	0
Cys	–	–	0
Val	1.04	1	1
Met	–	–	0
Ile	1.00	1	1
Leu	3.58	4	4
Tyr	–	–	0
Phe	0.96	1	1
Lys	1.87	2	2
His	1.67	2	2
Arg	1.73	2	2

Interaction between peptides

As shown in Table 2, αsl(1-23) and the pH 4.6-precipitated fraction had activities similar to intact αsl-casein when their concentrations were 2%. This result seems to be contradictory to the results of Fig. 1, in which the emulsifying activity of the pepsin digests of αsl-casein increased in step with the hydrolysis.

Consequently, the αsl(1-23) peptide was mixed with the pH 4.6-precipitated fraction at the primary ratio, and the emulsifying properties of this mixture were examined. The increase of EAI was confirmed in the mixture as seen in Table 3. It is considered that peptide/peptide interactions between αsl(1-23) and αsl(24-199) affect their emulsifying properties.

DISCUSSION

IN ORDER TO FIND a peptide having emulsifying activity,

Table 2—Emulsifying properties of αsl-casein, αsl(24-199) and αsl(1-23)-peptide[a]

	EAI[b] (m^2/g)	EC (mL oil/mg protein)	Adsorption (%)
αsl-casein	23.47 ± 0.98[c]	0.76 ± 0.014	9.76 ± 0.68
αsl(24-199)	23.58 ± 1.19	0.73 ± 0.046	5.53 ± 0.43
αsl(1-23)	23.19 ± 0.72	0.37 ± 0.010	72.54 ± 0.34

[a] Values are the average of three determinations
[b] Measured at the peptide concentration of 2% (w/w)
[c] Mean ± standard deviation

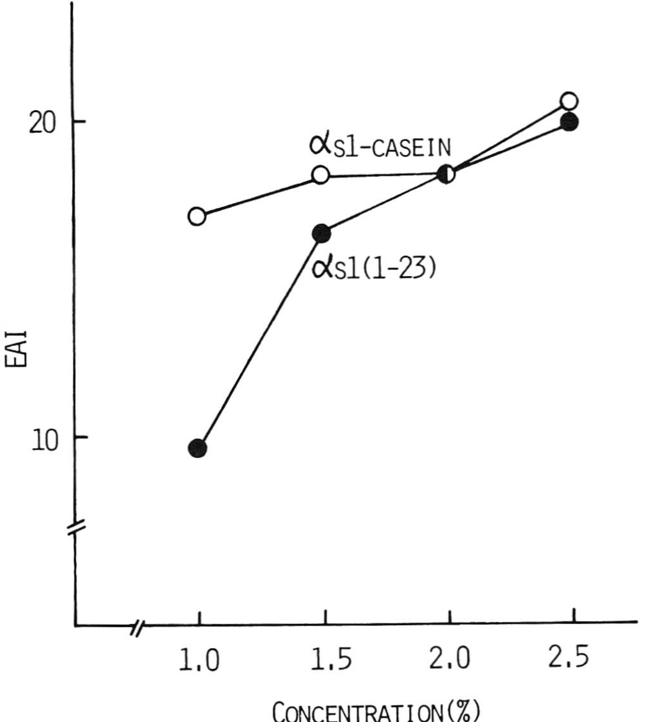

Fig. 4—Effect of concentration on the EAI of αsl-casein and αsl-(1-23) peptide.

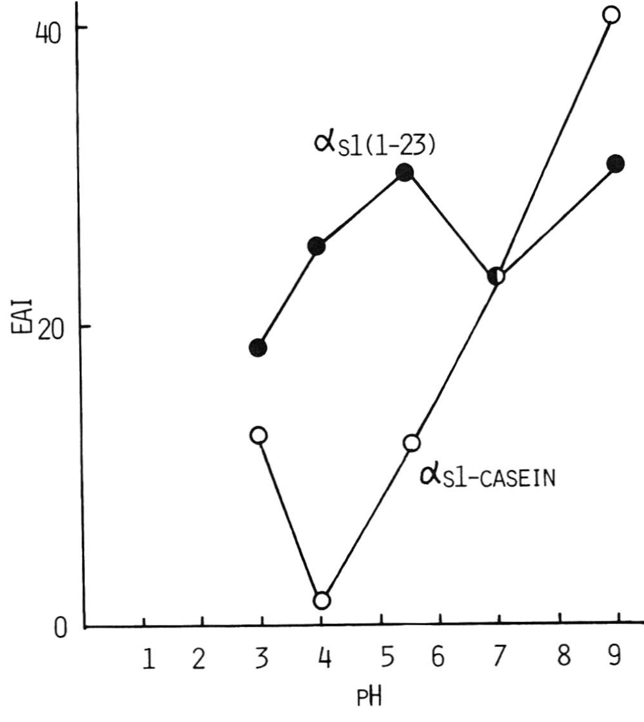

Fig. 5—Effect of pH on the EAI of αsl-casein and αsl(1-23) peptide. Each point is the average of two observations.

hydrolyzates of αsl-casein by some proteases were surveyed in this study. The chymosin and plasmin digestions caused no marked increase in the EAI of αsl-casein, while the pepsin digestion increased the EAI. αsl-casein was shown to be split into two peptides by pepsin digestion. The smaller peptide isolated from the digest was identified as αsl(1-23), and its emulsifying properties were investigated in detail.

αsl(1-23) showed a similar EAI to that of intact αsl-casein at neutral pHs, indicating that a peptide whose molecular weight is approximately 3000 can emulsify oil to a certain extent. However, the EC was about one-half compared with that of αsl-casein, and the amount of this peptide adsorbed onto an oil surface was markedly high. The remarkably high extent of adsorption and the low EC of αsl(1-23) suggest that the emulsification mechanism of this peptide is somewhat different from that of such proteins as αsl-casein.

On emulsification, proteins are adsorbed to the oil surface and spread to form a thin film covering the oil globules (Tornberg, 1980). The low EC might reflect a low spreadability of αsl(1-23).

On the other hand, the high adsorption might indicate that this peptide is highly interactive. Kaminogawa et al. (1980) have found that the hydrophobic N-terminal region of αsl-casein plays an important role in its intermolecular association by Ca^{++}. Creamer et al. (1982) have reported that the peptide segment involving residues 14-24 of αsl-casein contributes to the surface hydrophobicity of αsl-casein and plays an important role in the formation of a network of hydrophobically-bonded αsl-casein molecules in fresh cheese. Recently, Shimizu et al. (1983) have suggested that, on emulsification, αsl-casein was tightly adsorbed onto the oil globule surface, principally by its hydrophobic N-terminal region, and that it stabilized the oil globules. All of these findings suggest that αsl(1-23), the N-terminal peptide portion of αsl-casein, is highly interactive. The high extent of adsorption was probably because of the self-association of αsl(1-23) on the oil surface film as well as the interaction of this peptide with the oil phase.

The mixing of αsl(1-23) and the pH 4.6-precipitated fraction (αsl(24-199)) caused an increase of the EAI (Table 3). This synergistic effect might account for the increase in the EAI of αsl-casein during digestion by pepsin (Fig. 1). αsl(1-23) is a basic peptide containing 2 Arg, 2 Lys, 2 His and 2 Glu, and is positively charged at neutral pHs. Electrostatic interaction between this peptide and acidic αsl(24-199) might relate to such synergistic effect, though details on this kind of interaction remain obscure. Chymosin is known to produce αsl(1-23) and αsl(24-199) from αsl-casein like pepsin, but it further degrades αsl(24-199) during prolonged incubation (Mulvihill and Fox, 1979). The passing increase in EAI by chymosin treatment (Fig. 1) might also indicate that the two peptides, αsl(1-23) and αsl(24-199), produced at the first stage of chymosin digestion cooperatively take part in the high emulsifying activity.

Although the emulsifying properties of αsl(1-23) are not yet fully understood, and further studies are necessary to elucidate the adsorbing and spreading behavior of this peptide on the oil/water interface, the usage of this peptide in the food industry would seem to be promising. For example, αsl(1-23) maintains its solubility and emulsifying activity even in the acidic pH region. This property will be useful for the peptide to serve as an emulsifying agent in such foods with acidic pHs as processed cheese or fermented milk products.

Table 3—Emulsifying activity of αsl-casein and its peptide

	EAI[a] (m^2/g)
αsl-casein	21.10 ± 0.37[b]
αsl(24-199)	21.31 ± 0.81
αsl(1-23)	21.14 ± 0.22
a mixture of[c] αsl(24-199) + αsl(1-23)	23.13 ± 1.20

[a] Values are the average of three determinations measured at the peptide concentration of 2% (w/w) and at pH 6.4 (0.1M Na-acetate buffer)
[b] Mean ± standard deviation
[c] αsl(24-199) : αsl(1-23) = 86 : 14

Isolation and characterization of other functional peptides from αsl- and β-casein are now in progress.

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Freezing Time Prediction: An Enthalpy-Based Approach

SUDHIR K. SASTRY

ABSTRACT

Equations were developed for prediction of freezing times of foods of slab geometry, for three different boundary conditions, using a method of solving for enthalpy per unit volume instead of temperature. Experimental verification was performed for the operation with a convective heat transfer boundary condition, using ice cream and green peas in a granular packed bed as test materials. Results indicate good prediction of freezing times over a range of conditions. Results of thawing tests are less satisfactory if the ambient temperature lies in the zone of rapidly changing food properties. A comparison with literature predictions is presented.

INTRODUCTION

PREDICTION of freezing heat transfer in food products has received considerable interest and attention in recent years. Of particular interest has been the determination of the time requirement for the freezing and thawing of foods. It has been well established (Bonacina et al., 1973; Bakal and Hayakawa, 1973; Heldman, 1974), that food products do not freeze at a single temperature, but over a range of temperatures below the initial freezing point. In addition, the thermal properties undergo marked changes upon transition of water from the liquid to the solid phase. Thus, the resulting heat transfer problem is one of considerable complexity.

Numerous approaches have been used to obtain solutions to the heat transfer problem. These may be classed into two broad categories: (1) analytical or approximate analytical solutions, and (2) numerical solutions.

Analytical solutions have the principal advantage of simplicity and ease of use; however, simplifying assumptions used in obtaining the analytical results may lead to inaccuracies in certain cases. The simplest relation is that of Plank (1941), which provides an explicit expression for the freezing time of a pure ice-water system, involving a single freezing point. Plank's relation has been modified and improved by numerous researchers including Nagaoka et al. (1955), Cleland and Earle (1979), Hung and Thompson (1983), Scott and Hayakawa (1977), Mascheroni and Calvelo (1982), and others. Other analytical approaches have involved the use of Goodman's heat balance integral method (Goodman, 1958) which is particularly useful in phase-change problems. Solutions have been obtained by Tien and Geiger (1967, 1968) and Tien and Koump (1968), for the solidification of alloys, and by Hayakawa and Bakal (1973), and Bakal and Hayakawa (1973) for foods. These relations have provided information on temperature as a function of location in the product and time.

Numerical solutions of considerable sophistication have been obtained by many researchers; including Bonacina et al. (1973), Heldman (1974), Heldman and Gorby (1975), Hsieh et al. (1977), Schwartzberg et al. (1977), DeMichelis and Calvelo (1982), and Tao (1967). Numerical solutions are sophisticated, possess fewer simplifying assumptions than analytical solutions, and are therefore more accurate in their depiction of temperature profiles and histories in foods undergoing freezing. However, they are less easy to use than analytical solutions, and require knowledge of numerous material properties. Tao's (1967) solutions, however, are available in the form of charts, which facilitate their use.

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The present research is an attempt to combine convenience with accuracy. This is done by transforming the governing heat transfer equations so that enthalpy per unit volume is used as the dependent variable instead of temperature. This results in 'enthalpy profiles' instead of temperature profiles. Since enthalpy of the food product is a single-valued function of temperature, the solution of any freezing time problem by this method requires the use of enthalpy-temperature data (such as presented by ASHRAE, 1981) for the food product under consideration. Solutions presented in this paper are sought in the form of polynomials. Accuracy may be improved by use of polynomials of higher order; however, this increases the complexity of the solution.

The objective of this research was to develop explicit expressions for freezing time, while retaining the considerations that freezing occurs over a wide temperature range, and that thermal properties are temperature-dependent.

MATERIALS & METHODS

Derivation of formulae

Heat transfer analyses are performed for an infinite slab, initially of uniform temperature, and in contact with the freezing medium on two opposite sides. By symmetry, the problem may be reduced to that of heat transfer through a slab, one-half as thick, with one side insulated, as shown in Fig. 1. The following three cases are considered in obtaining solutions.

Case I. A constant temperature condition at the boundary of the food product. This situation occurs when the material is contact-frozen in a plate freezer, where the slab of material is in contact with cold plates of large thermal mass and contact resistance is negligible. Such a situation would also occur when the food product is blast-frozen, and high Biot Numbers exist.

Case II. A constant heat flux at the boundary. This condition is approximated when cyrogenic freezing is done; the temperature of boiling liquid being so low as to make little difference in the heat flux throughout the freezing process.

Case III. A convective condition at the boundary. This is a more realistic depiction of most freezing situations, when Biot numbers are in an intermediate zone, thereby permitting neither an application of Case I, nor the use of Newton's law of cooling.

In all of the above cases, freezing is considered to occur in two stages. In stage I (illustrated in Fig. 2a for case I) the temperature disturbance penetrates a distance τ into the material, while that part of the material located further away from the surface, is entirely undistrubed. Stage I ends when the disturbance reaches the center of the slab, (or the insulated face in the modified problem). In stage II (Fig. 2b for case I), the temperature of the center drops. This continues until the center of the product achieves the desired temperature, at which time freezing is considered concluded. Similar stages are considered to occur for cases II and III as well.

It has been generally recognized (Boancina et al., 1973; Heldman, 1974) that the problem of heat transfer with phase change can be reduced to one of simple conduction heat transfer with temperature-dependent thermophysical properties. This is done by lumping all

latent heat effects with the heat capacity to form an 'effective' heat capacity term, which is the derivative of enthalpy with respect to temperature. Using this approach, the concept of a 'freezing front', can be left undefined (since foods are never completely frozen, no sharply defined freezing fronts exist). For the present case of one-dimensional conduction involving Cartesian coordinates:

$$\frac{\partial}{\partial x}\left[k(T)\frac{\partial T}{\partial x}\right] = \rho\frac{dh}{dT}\frac{\partial T}{\partial t} \tag{1}$$

with the initial condition specifying the temperature datum.

$$T(x,0) = 0 \tag{1a}$$

Eq. (1) may be transformed into one involving enthalpy per unit volume (u), by using the following definition:

$$u = \int_0^T \rho \frac{dh}{dT}\, dT = \int_0^h \rho\, dh \tag{2}$$

Considering u as function of T as above, and employing chain rules of differentiation, the variable T may be replaced by u in Eq. (1) yielding

$$\frac{\partial}{\partial x}\left[\alpha(u)\frac{\partial u}{\partial x}\right] = \frac{\partial u}{\partial t} \tag{3}$$

where $\alpha(u) = \alpha(u(T)) = k(T)/(\rho \frac{dh}{dT})$

Further, the datum for u is defined to correspond to that at the initial condition (1a). Thus,

$$u(x,0) = 0 \tag{3a}$$

It is important to note that all values of u are considered in relation to that of Eq. (3a).

Next, the formulations are obtained for each stage, using Goodman's heat balance integral approach. For stage I, regardless of boundary condition, Eq. (3) is integrated between 0 and τ. The resulting equation is simplified using the Leibnitz rule and by imposing the constraint that

$$\frac{\partial u(\tau,t)}{\partial x} = 0$$

to yield the governing equation

$$-\alpha(u)\frac{\partial u(0,t)}{\partial x} = \frac{d}{dt}\int_0^\tau u\, dx \tag{4}$$

For stage II, the treatment is identical except that the integration is between 0 and L/2.

$$-\alpha(u)\frac{\partial u(0,t)}{\partial x} = \frac{d}{dt}\int_0^{L/2} u\, dx \tag{5}$$

Thus, the formulations for each case are as follows.

Case I: Constant temperature boundary condition

In stage I, Eq. (4) is the governing equation, with conditions

$$u(0,t) = u_s \tag{4a}$$

$$u(\tau,t) = 0 \tag{4b}$$

$$\frac{\partial u(\tau,t)}{\partial x} = 0 \tag{4c}$$

In stage II, Eq. (5) is the governing equation, with conditions

$$u(0,t) = u_s \tag{5a}$$

$$\frac{\partial u(L/2,t)}{\partial x} = 0 \tag{5b}$$

A third condition is obtained by equating stage I and stage II profiles at the time of transition from one stage to the other.

Case II: Constant heat flux boundary condition

Again, in stage I, Eq. (4) is used, along with

$$-\alpha(u)\frac{\partial u(0,t)}{\partial x} = q = \text{constant} \tag{4d}$$

$$u(\tau,t) = 0 \tag{4e}$$

$$\frac{\partial u(\tau,t)}{\partial x} = 0 \tag{4f}$$

In stage II, Eq. (5) is the governing equation, with conditions

$$-\alpha(u)\frac{\partial u(0,t)}{\partial x} = q \tag{5d}$$

$$\frac{\partial u(L/2,t)}{\partial x} = 0 \tag{5e}$$

Again, stage I and stage II profiles are equated at the time of transition to obtain a third condition.

Case III: Convective boundary condition

In stage I, Eq. (4) is used, along with

$$-\alpha(u)\frac{\partial u(0,t)}{\partial x} = h_c(T_\infty - T_s) \tag{4g}$$

$$u(\tau,t) = 0 \tag{4h}$$

$$\frac{\partial u(\tau,t)}{\partial x} = 0 \tag{4j}$$

Condition (4g) is modified further by the following simplifying assumptions:

(1) The surface of the food product rapidly reaches a temperature sufficiently close to the ambient environment; such that little variation occurs in the thermal diffusivity at the surface of the mate-

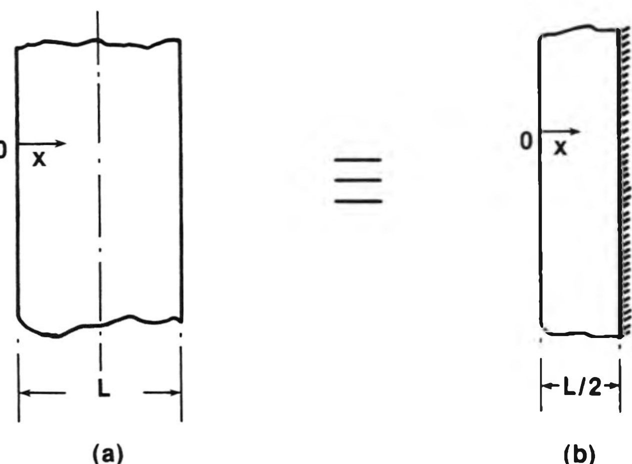

Fig. 1—Equivalent problem statements: (a) Symmetric infinite slab. (b) Slab of half-thickness insulated at L/2.

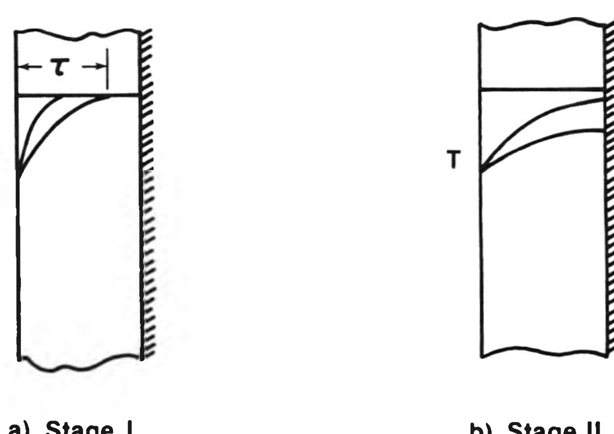

Fig. 2—Stages of case I: (a) Temperature disturbance has not reached center of slab. (b) Temperature distribution has reached center of slab.

rial for the remainder of the freezing period. This assumption is justifiable in a freezing situation, since thermal properties of foods undergo relatively minor changes at low temperatures. Thus, the left hand side of Eq. (4g) becomes

$$-\alpha(u)\frac{\partial u(0,t)}{\partial x} = -\alpha_s\frac{\partial u(0,t)}{\partial x} \quad (4k)$$

where α_s = constant.

(2) The enthalpy-temperature curve for the food material approaches a straight line at temperatures close to the ambient value. This assumption is reasonably accurate at low temperature where a large portion of the water in the food product is frozen. Thus, it can be applied to freezing situations. The right hand side of Eq. (4g) can then be rewritten as

$$h_c[T_\infty - T(0,t)] = h_c\gamma[u_\infty - u(0,t)] \quad (4l)$$

where $\gamma = dT/du$ in the zone of constant slope. Thus, condition (4g) can be modified to the following

$$-\alpha_s\frac{\partial u(0,t)}{\partial x} = h_c\gamma[u_\infty - u(0,t)] \quad (4g)$$

The effect of simplifying assumptions on prediction accuracy is discussed in the results section of this paper.

In stage II of case III, Eq. (5) is the governing equation, with conditions

$$-\alpha_s\frac{\partial u(0,t)}{\partial x} = h_c\gamma[u_\infty - u(0,t)] \quad (5f)$$

$$\frac{\partial u(L/2,t)}{\partial x} = 0 \quad (5g)$$

As in all earlier cases, stage I and stage II solutions are equated at the transition point to obtain the third condition.

The solution procedure for each of the three cases involves seeking enthalpy profiles in the form of second degree polynomials. Details of the procedure are provided in the appendix for Case III only. The procedures for cases I and II are identical.

Using the criterion that the food product is frozen when the enthalpy at L/2 reaches the desired value, u_f, the following freezing time formulae are obtained.

Case I:

$$t_f = \frac{L^2}{12\alpha_s}\left\{\frac{1}{4} - \ln(1 - \frac{u_f}{u_s})\right\} \quad (6)$$

Case II:

$$t_f = \frac{L}{2\alpha_s}(\frac{\alpha_s u_f}{q} + \frac{L}{12}) \quad (7)$$

Case III:

$$t_f = t_I - \frac{1}{\beta}\ln(\frac{u_\infty - u_f}{u_\infty}) \quad (8)$$

where t_I = time taken for stage I

$$= \frac{1}{6\alpha_s}\left\{\frac{L^2}{8} + \frac{\alpha_s L}{h_c\gamma} + \frac{4\alpha_s^2}{h_c^2\gamma^2}\ln(\frac{2\alpha_s}{h_c\gamma(\frac{L}{2} + \frac{2\alpha_s}{h_c\gamma})})\right\} \quad (9)$$

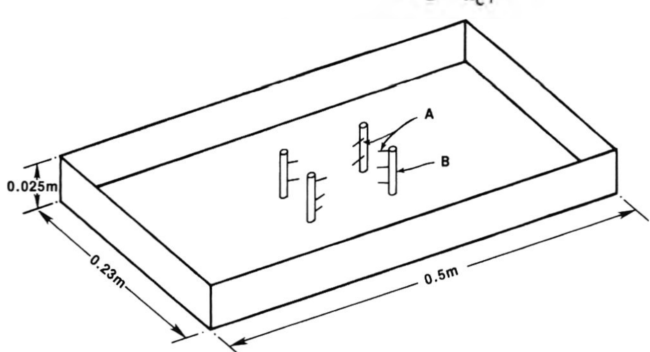

Fig. 3—Diagram of slab-shaped container used in experiments, showing: (A) Thermocouple junctions; (B) Insulated inlet tubes.

and

$$\beta = \frac{12h_c\gamma\alpha_s}{L(6\alpha_s + h_c\gamma L)} \quad (10)$$

It is important to note that all values of u are defined with the initial condition representing the datum, as in Eq. (3a). Further, the variable u refers to enthalpy per unit volume. Since most tables such as presented by ASHRAE (1981) provided data on enthalpy per unit mass, and use a datum point of −40°C, it is necessary to know the material density and to convert to a datum corresponding to the initial temperature of the material.

The proposed equations can be extended to a precooling situation, where the initial temperature of the product lies above the initial freezing point. The enthalpy-temperature curve for the food product can be extended to values above freezing, by multiplying the temperature above freezing by the specific heat of the unfrozen material. By extension of the curve, Eq. (6), (7) and (8) can be used for precooling situations.

Experimental verification

Experiments for freezing and thawing were conducted on two different test materials; ice cream and green peas in a packed bed. Tests were performed primarily for verification of the case III relation, represented by Eq. (8), since this represents a more commonly encountered situation than either case I or II. All tests were conducted using slab-shaped containers made of galvanized sheet steel, similar to those illustrated in Fig. 3. The thickness was made much smaller than the length and width so that one-dimensional heat transfer could be approximated. Thermocouples were located at 11 points across the thickness of the slab, spaced apart at distances equal to 1/10th the thickness. To eliminate effects of a single location with respect to the length and width, inlet tubes were provided at four different locations on the base. Thus, any variations of temperature due to location with respect to the length and width were monitored. All inlet tubes were insulated to protect thermocouples and prevent errors due to conduction along the thermocouple wire length. Further, each of the tubes was placed sufficiently close to the center of the base to minimize end effects.

Ice cream freezing tests were conducted over the temperature range associated with the hardening stage following initial slush freezing in scraped-surface heat exchangers. Samples, obtained from the Pennsylvania State University Creamery were of vanilla flavor, containing no particulate additives, and consisted of 13.0% fat, 11.0% nonfat milk solids, 13.0% cane sugar, 3.7% corn syrup solids, 0.25% stabilizer and 59.05% water. Samples were tested at three different overrun levels: 50, 80 and 100%. Bulk densities were measured at the time of the tests; and these corresponded to 780 kg/m^3 (50% overrun), 683 kg/m^3 (80% overrun) and 634 kg/m^3 (100% overrun).

In all freezing experiments, the slab-shaped container was filled with ice cream, and placed in a horizontal position within a freezer set at −4°C. The slab rested on an insulated base, to minimize heat transfer from the bottom end, and to approach the condition of Fig. 1b. When thermal equilibration occurred, the box, together with the insulated base, was quickly transferred to a freezer at −23°C. Temperatures were recorded every 15 minutes, as the sample cooled by natural convection. When equilibrium was attained, the sample was transferred back into the freezer at −4°C for thawing experiments. Temperatures were recorded at 15-min intervals. In an effort to test the effect of slab thickness, samples at 100% overrun were also tested in a slab of thickness 0.051m.

For freezing tests with green peas, the sample was packed into the slab at a bulk density of 556 kg/m^3, and then covered with a lid and sealed to prevent air infiltration. The slab was placed within a freezer at −4°C in a vertical position to prevent natural convection effects across the thickness. Thus no insulation base was used, and the situation approached that described in Fig. 1a. Upon equilibration, the slab was quickly transferred to a freezer at −23°C, where it was again placed in a vertical position. For all freezing tests with peas, the air within the freezer was agitated by blowers. Temperatures were recorded every 15 minutes. After equilibration, the slab was once again transferred to the freezer at −4°C and placed in a vertical position for thawing tests. All thawing experiments were conducted under natural convection conditions.

For comparison of experimental results to theoretical predictions, the properties, ρ, h_c and k, must be known in addition to the curve of u versus T. Density (ρ) was measured by weighing the sam-

ples in the slab and measuring the slab volume. The convective heat transfer coefficient (h_c) was determined from the Nusselt number, using the procedures described as follows.

For freezing and thawing of ice cream, the relations used were those for free convection from horizontal flat surfaces, (Perry and Chilton, 1973).

$$Nu = 0.54(Gr\ Pr)^{0.25} \quad (11)$$

for $10^5 < Gr{\cdot}Pr < 2 \times 10^7$, and

$$Nu = 0.14(Gr\ Pr)^{0.333} \quad (12)$$

for $2 \times 10^7 < Gr{\cdot}Pr < 3 \times 10^{10}$.

For freezing of peas, (forced convection) the heat transfer coefficient was determined experimentally by using the data of the first freezing run, and varying the parameter h_c in Eq. (8) until satisfactory prediction was obtained. This value was then used in calculations for all other runs of the experiment. The thermal resistance of the slab material was determined by dividing the thickness of the sheet steel by the thermal conductivity, and was found to be negligible in comparison to the boundary layer resistance.

For thawing of peas, the relation presented by Perry and Chilton (1973) for natural convection from a vertical flat surface was used.

$$Nu = 0.59\ (Gr\ Pr)^{0.25} \quad (13)$$

for $10^4 < Gr{\cdot}Pr < 10^9$.

Thermal conductivity of peas and ice cream were determined at various temperatures and bulk densities using the line source thermal conductivity probe method of Nix et al. (1967). Details are provided by Sastry and Datta (1983). Data on enthalpy of peas and ice cream as functions of temperature were obtained using an electrically heated calorimeter wherein a known quantity of samples was heated, and the power input and temperature rise are recorded. A detailed description is provided by Sastry and Datta (1983).

RESULTS & DISCUSSION

IN THE ICE CREAM freezing tests, thermal equilibria were attained in 9-11 hr, while thawing times ranged from 9.5-13 hr depending on the experimental conditions. In tests on peas, equilibration during freezing was attained in 2-3 hr, while thawing equilibria occurred in approximately 7 hr. In all cases, the time t_I till the end of stage I was found to be small – in the order of 1-2 min. This was consistent with theoretical predictions of t_I.

Since the criterion for definition of freezing time is dependent on the choice of the final center temperature, T_f, it is necessary to present results over a wide range of T_f values. Since the conditions of each replication were slightly different, the initial, final and center temperatures are expressed by using a dimensionless temperature ratio (TR) defined as

$$TR = (T_i - T_f)/(T_i - T_\infty) \quad (14)$$

The temperature ratio has a value of zero at the beginning of the freezing or thawing process; and reaches a value of unity when equilibrium with the environment is achieved.

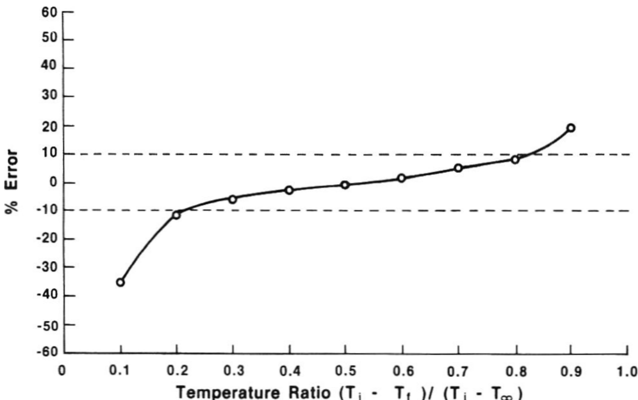

Fig. 4—Error vs temperature ratio for freezing of ice cream.

The average percent error (from seven replications) in prediction of freezing time for ice cream is plotted against TR in Fig. 4. The results show good prediction (±10% error) over a wide range (0.25-0.83) of TR values which may be expected to occur in most commercial freezing operations. Error for very low values of TR fall in the negative range and those for high TR values fall on the high positive side. The maximum standard deviation of errors is 4.45%.

Errors in the low range of TR values may be due to the first simplifying assumption made in derivation; that the surface reaches a temperature close to the ambient value such that little variation in surface thermal diffusivity occurs for the remainder of the freezing period. This appears to be a good assumption if the T_f value is not too high; however, in freezing experiments, the surface temperature does not immediately attain the ambient value, but approaches it at a rapid rate. In the early stages of the freezing process, the surface of the product is at a higher temperature than the ambient value, though it is assumed to be equal to the ambient. Referring to a typical enthalpy-temperature curve, such as that shown in Fig. 5, the slope estimated is lower than the actual value. A low estimate of the surface slope results in a lowered prediction from Eq. (8); thus the negative nature of the error. As the TR value increases, the surface temperature assumption becomes more and more valid, resulting in improved prediction.

At TR values approaching unity, the large positive errors associated with any exponential solution occur. However, this does not occur until the TR value is close to unity. The results provide good prediction of freezing time within the range of TR values encountered in most commercial freezing situations. For example, ice cream hardened from −4°C

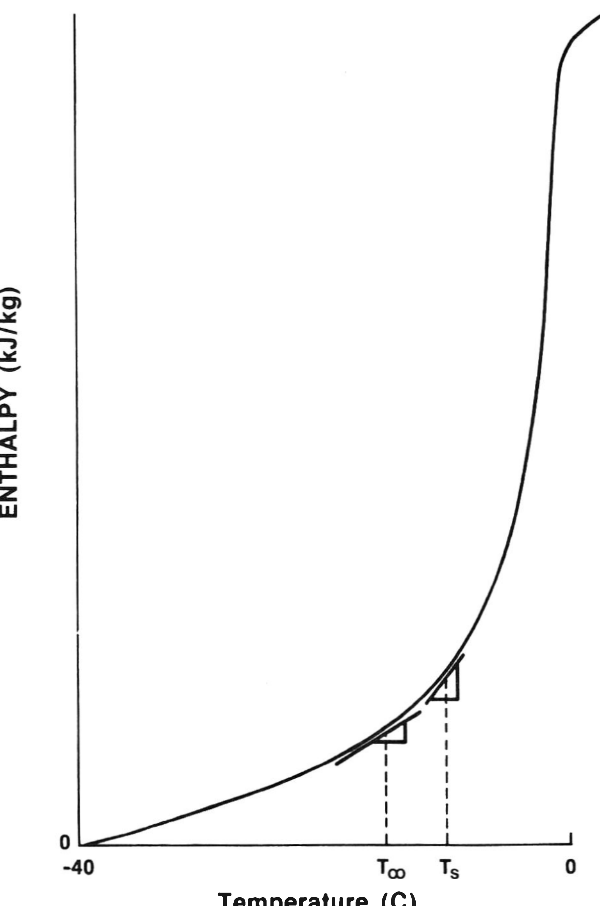

Fig. 5—Typical enthalpy-temperature curve for foods.

to $-18°C$ using air at $-30°C$ has a TR value of 0.54; well within the range of satisfactory prediction.

Errors in prediction for freezing of peas in a packed bed are shown in Fig. 6 (average of 3 replications). Results show very good prediction over an even wider range than for ice cream. Maximum standard deviation of errors is 5.3%. The trends in the errors, however, are not the same. This may be due to the freer air movement possible in a packed-bed granular system than in a frozen emulsion such as ice cream where air bubbles are trapped and not free to move.

Errors in prediction for the thawing case; both for ice cream (7 replications) and for peas (3 replications) are high, as evidenced by Fig. 7 and 8. The maximum standard deviation of errors is 20.66% for ice cream and 26.30% for peas. The reason appears to be the second simplifying assumption, that the slope of the enthalpy-temperature curve approaches a constant value at temperatures close to ambient. The temperature chosen in this case was $-4°C$, which lies in the zone of very large slope changes in the enthalpy temperature curve (Fig. 5), both for peas and for ice cream. This would result in considerable overestimation of the slope when the first assumption is used, and large positive errors would occur. It remains to be investigated whether this error would occur if thawing temperatures were above freezing, since the enthalpy-temperature curve would approach a slope equal to the specific heat of the material at ambient conditions.

Predictions from this research were compared to other analytical methods in the literature, specifically those of Plank (1941), Cleland and Earle (1977) and Hung and Thompson (1983). Values of parameters used are listed in Table 1. Results of comparison are presented in Table 2 for various values of temperature ratio (TR). It is noted that Plank's formula consistently yields a low prediction. Predictions of Cleland and Earle (1977) and Hung and Thompson (1983) yield large positive errors at low values of TR, and negative errors at high TR values. Cleland and Earle's method yields satisfactory prediction between TR values of approximately 0.5 and 0.7; while that of Hung and Thompson yields good prediction in the vicinity of a TR value of 0.8. It is notable that both these formulae are based on Plank's (1941) formula, which assumes that freezing occurs at a single temperature. In ice cream hardening situations, this assumption may have some limitations. The results of Table 2 indicate the importance of the influence of temperature ratio (TR) on the errors involved in freezing time prediction. Much of the current literature on analytical freezing time prediction methods does not specify this parameter; but its influence on errors in heat transfer problems merits consideration. The value of temperature ratio can help in determining the relative influence of the nonlinearity in specific freezing situations. Nonlinearity appears significant for small values of temperature ratio, but its influence decreases as TR increases.

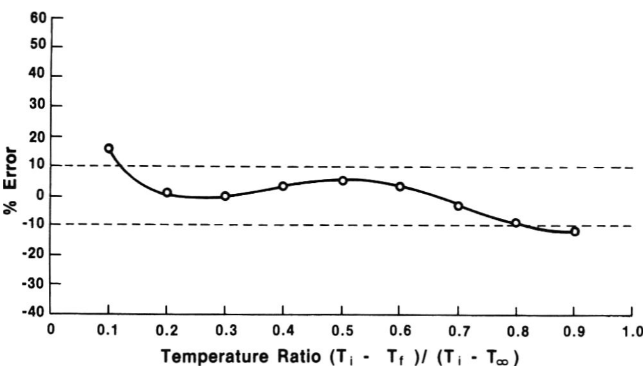

Fig. 6—Error vs temperature ratio for freezing of green peas in a granular packed bed.

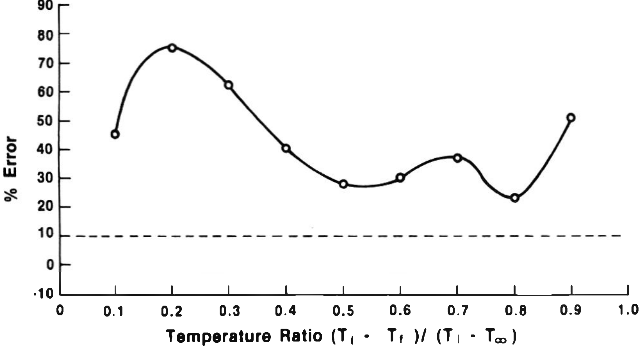

Fig. 7—Error vs temperature ratio for thawing of ice cream.

CONCLUSIONS

Freezing time equations have been developed for foods of slab geometry, for three different boundary conditions, using a method of solving for enthalpy instead of temperature.

The equation developed for the convective heat transfer boundary condition, gives good prediction for the freezing times of ice cream and green peas in a packed bed, over a range of conditions.

Thawing rate predictions are less satisfactory than freezing rate predictions if the ambient temperature lies in the range of rapidly changing properties.

The equations provided have the potential of being used both in precooling and freezing situations, if the enthalpy-temperature curve is extended sufficiently.

The equations require knowledge of material thermal conductivity at only one temperature; the ambient. However, the enthalpy-temperature data for the food product must be known.

The temperature ratio is an important factor influencing the accuracy of freezing time calculations.

APPENDIX

Derivation of solution for case III

Recall that for stage I, the governing equation is:

$$-\alpha(u)\frac{\partial u(0,t)}{\partial x} = \frac{d}{dt}\int_0^{\tau} u\,dx \qquad \text{(A1)}$$

with conditions

$$-\alpha_s\frac{\partial u(0,t)}{\partial x} = h_c\gamma(u_\infty - u(0,t)) \qquad \text{(A2)}$$

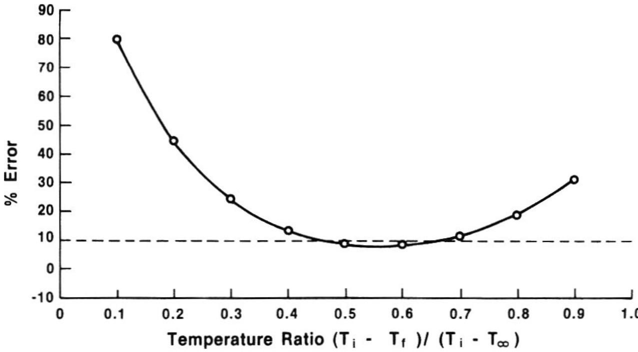

Fig. 8—Error vs temperature ratio for thawing of green peas in a granular packed bed.

Table 1—Parameter values used in prediction of freezing times for ice cream

Parameter	Symbol	Value or Range of Values
Thermal conductivity[a]	k	0.00053-0.00059 kw/m C
Enthalpy change[a]	Δh	5.33-112.46 kJ/kg
Density	ρ	597-780 kg/m^3
Slab thickness	L	0.0254m, 0.0508m
Convective coefficient	h_c	From Eq. 11-13
Initial temperature[a]	T_i	−2.6 to −6.4c
Air temperature[a]	T_∞	−20 to −23.6c

[a] The exact value used depends on the experimental conditions or on the value of temperature ratio (TR) used to designate the final frozen state.

$$u(\tau,t) = 0 \qquad (A3)$$

$$\frac{\partial u(\tau,t)}{\partial x} = 0 \qquad (A4)$$

The solution is sought in the form of a second-degree polynomial,

$$u = a + bx + cx^2 \qquad (A5)$$

where a, b, and c are functions of time. They are determined by applying conditions (A2), (A3), and (A4) to Eq. (A5). This results in

$$a = \frac{\epsilon u_\infty}{(1+\epsilon)} \qquad (A6)$$

$$b = \frac{2\epsilon u_\infty}{\tau}\left\{\frac{\epsilon}{(1+\epsilon)} - 1\right\} \qquad (A7)$$

$$c = -\frac{\epsilon}{\tau^2}\left\{\frac{\epsilon}{(1+\epsilon)} - 1\right\} \qquad (A8)$$

where $\epsilon = (h_c\gamma\tau)/(2\alpha_s)$, and τ is an unknown function of t. Substituting the value of u from Eq. (A5) back into (A1) and solving, yields the following relation between τ and t.

$$\frac{\tau^2}{2} + \theta\tau + \theta^2 \ln\left\{\frac{\theta}{(\tau+\theta)}\right\} = 6\alpha_s t \qquad (A9)$$

where $\theta = (2\alpha_s)/(h_c\gamma)$. Eq. (A9) does not yield an explicit relation between τ and t. However, the time t_I until the end of stage I may be readily determined, by applying the condition

$$\tau(t_I) = L/2$$

Thus

$$t_I = \frac{1}{6\alpha_s}\left\{\frac{L^2}{8} + \frac{\theta L}{2} + \theta^2 \ln\left(\frac{\theta}{(L/2+\theta)}\right)\right\} \qquad (A10)$$

For stage II, the governing equation is

$$-\alpha(u)\frac{\partial u(0,t)}{\partial x} = \frac{d}{dt}\int_0^{L/2} u\,dx \qquad (A11)$$

with conditions

$$-\alpha_s\frac{\partial u(0,t)}{\partial x} = h_c\gamma[u_\infty - u(0,t)] \qquad (A12)$$

$$\frac{\partial u(L/2,t)}{\partial x} = 0 \qquad (A13)$$

Again, a solution is sought in the form of a second degree polynomial similar to that of Eq. (A5).

$$u = a' + b'x + c'x^2 \qquad (A14)$$

where the coefficients are unknown functions of t. Applying conditions (A12) and (A13), two of these coefficients, b′ and c′ may be determined as functions of a′. To determine a′ as a function of t, the resulting polynomial is applied to the governing equation, (A11), and the resulting equation solved. The following expressions are obtained

Table 2—Comparison of errors in freezing time prediction for ice cream

TR	Plank (1941)	Cleland and Earle (1977)	Hung and Thompson (1983)	This work
	Average error (%)[a]			
0.1	−29.5	165.5	482.7	−36.0
0.2	−16.6	81.7	225.9	−11.2
0.3	−12.0	40.3	97.0	− 7.4
0.4	−12.5	22.2	44.5	− 2.0
0.5	−16.0	13.4	29.0	− 0.3
0.6	−21.0	4.9	23.4	1.2
0.7	−27.1	− 7.0	13.1	5.6
0.8	−34.4	−21.0	− 4.2	9.1
0.9	−44.1	−30.9	−18.5	19.3

[a] Average for 7 replications

$$b' = \frac{h_c\gamma}{\alpha_s}(a' - u_\infty) \qquad (A15)$$

$$c' = \frac{h_c\gamma}{\alpha_s L}(a' - u_\infty) \qquad (A16)$$

and

$$a' = C\exp(-\beta t) + u_\infty \qquad (A17)$$

where β is given by Eq. (10), and C is a constant of integration to be determined by using a third condition to the governing equation. This condition is that at t_I, the time of transition between stages, the stage I and stage II profiles are identical. Thus, substituting values for a, b, c, and a′, b′ and c′ into Eq. (A5) and A14), respectively, and comparing coefficients, the value of C is found.

$$C = -\frac{u\exp(\beta t_I)}{\left(1 + \frac{h_c\gamma L}{4\alpha_s}\right)} \qquad (A18)$$

Thus the stage II profile is completely determined.

Using the criterion that the product is considered frozen when the center temperature reaches a predefined value, T_f corresponding to an enthalpy u_f, the freezing time is

$$t_f = t_I - \frac{1}{\beta}\ln\left(\frac{u_\infty - u_f}{u_\infty}\right) \qquad (A19)$$

NOMENCLATURE

- a coefficient in Eq. (A5)
- a′ coefficient in Eq. (A14)
- b coefficient in Eq. (A5)
- b′ coefficient in Eq. (A14)
- c coefficient in Eq. (A5)
- c′ coefficient in Eq. (A14)
- Gr Grashof number
- h enthalpy (kJ/kg)
- h_c convective heat transfer coefficient (w/m^{2}C)
- k thermal conductivity (w/m C)
- L slab thickness (m)
- Nu Nusselt number
- Pr Prandtl number
- q heat flux (w/m^2)
- t time (s)
- t_f freezing time (s)
- t_I stage I duration (s)
- T temperature (C)
- T_f temperature at frozen condition (C)
- T_i initial temperature (C)

T_s surface temperature (C)
T_∞ ambient temperature (C)
TR temperature ratio
u enthalpy per unit volume (J/m^3)
u_f enthalpy per unit volume, frozen condition (J/m^3)
u_s enthalpy per unit volume, surface condition (J/m^3)
u_∞ enthalpy per unit volume, ambient condition (J/m^3)
x length coordinate (m)

Greek letters

α thermal diffusivity (m^2/s)
α_s thermal diffusivity, surface condition (m^2/s)
β parameter in Eq. (10) (s^{-1})
γ reciprocal slope of u-T curve ($C\ m^3/J$) (defined in Eq. 41)
ϵ parameter defined in Eqs. (A6), (A7) and (A8)
ρ density of product (kg/m^3)
θ parameter defined in Eq. (A9)
τ distance of penetration of thermal disturbance into the food product (m)

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Functional Properties of Acylated Oat Protein

CHING-YUNG MA

ABSTRACT

Protein extracted from defatted oat (*Avena sativa* L., variety Sentinel) was acylated with acetic or succinic anhydride at levels of 0.05 and 0.20 g/g protein. Acetic anhydride was more reactive than succinic anhydride in modifying lysine groups. Total essential amino acid content was slightly lowered by acetylation but unaffected by succinylation. Gel filtration chromatography showed some dissociation of oat polypeptides by succinylation. Solubility, emulsifying properties and fat finding capacity were all markedly improved by acylation, and the effect was more pronounced with succinylation. Emulsifying capacity of meat was enhanced by blending with acylated oat protein. Water hydration capacity and foam stability were adversely affected by acylation. Results suggest that acylated oat protein may be a valuable functional ingredient in meat and other emulsion food products.

INTRODUCTION

THE STORAGE PROTEINS from oats are nutritionally superior to those of other cereals, having a balanced amino acid profile and no antinutritional or toxic components. However, oat proteins are not used extensively for human consumption, partly due to a lack of knowledge on their functional behavior in processed foods. In previous papers (Ma, 1983a, b), the functional properties of oat protein concentrates and isolates have been described. Although oat protein products were shown to possess good emulsifying and binding properties, it would be desirable to further improve these and other functional properties, particularly solubility which is poor at neutral and slightly acidic pH (Ma, 1983a). This will enhance the value of oat protein as a food ingredient. Among various physical and chemical methods used to improve protein functionality, acylation is one of the most effective, and has been applied to many plant proteins including wheat (Grant, 1973), soybean (Franzen and Kinsella, 1976), cottonseed (Childs and Park, 1976; Choi et al., 1981), peanut (Sundar and Rao, 1978; Beuchat, 1977), sunflower (Canella et al., 1979; Kabirulah and Wills, 1982), and pea (Johnson and Brekke, 1983). The purpose of this study was to acylate oat protein by either acetic or succinic anhydride, and determine some chemical and functional properties of the modified protein.

MATERIALS & METHODS

Materials

Oats (*Avena sativa* L., variety Sentinel) were grown in the Central Experimental Farm, Ottawa, Canada in 1981. The seeds were dehulled and ground in a pin-mill, and then defatted by Soxhlet extraction with hexane.

Methods

Preparation of acylated oat protein. Proteins from the defatted oat groats were extracted with a weak alkali (0.015N NaOH) as described previously (Ma, 1983a). The slurry was stirred at room temperature for 60 min and then centrifuged at 4,000 X *g* to remove the starch. Acetic or succinic anhydride was added to the supernatant at levels of 0.05 and 0.20g per g protein, and the pH was maintained at 8.0 by adding 1N NaOH. The supernatant was then dialyzed against distilled water at 4°C for 48 hr and freeze-dried.

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Chemical analysis. The extent of acylation was estimated from the free lysine content of the acylated and unmodified oat protein samples, using the dinitrobenzene sulfonate (DNBS) method of Concon (1975). Protein contents were determined by the micro-Kjeldahl analysis (Concon and Soltess, 1973), using a nitrogen to protein conversion factor of 6.25. Amino acid analysis were performed according to Spackman et al. (1958).

Gel filtration chromatography. Gel filtration chromatography was performed on a 2.5 X 45 cm column of Sephacryl S-200 (Pharmacia Fine Chemicals, Uppsala, Sweden). The buffer used was 2 M sodium thiocyanate as described by Preston (1982). The column was calibrated with standard proteins of known molecular weight, including human γ-globulin, bovine serum albumin, ovalbumin, trypsin inhibitor and cytochrome C.

Functional properties. Solubility was determined in 1% dispersions at pH between 1.5 and 10.0 as described previously (Ma, 1983a). Emulsifying capacity (EC) was determined by the procedure of Swift et al. (1961), using 0.01M potassium phosphate buffer, pH 7.4. The EC of mixtures of meat (beef round) and oat proteins was also determined. Meat was homogenized with a Polytron in 1M NaCl to produce a 1% suspension of muscle proteins, and was mixed with the oat protein samples at various ratios from 0–100%. A turbidimetric method (Pearce and Kinsella, 1978) was used to assess the emulsifying activity and emulsion stability of oat proteins. Water hydration capacity (WHC) was determined by the method of Quinn and Paton (1979). Fat binding capacity (FBC) was determined according to Lin et al. (1974). The foaming capacity and stability were assessed by the procedure of Yatsumatsu et al. (1972).

In vitro digestibility. A multienzyme method (Hsu et al., 1977) was used to determine the in vitro digestibility of acylated oat protein. Trypsin (type IX from porcine pancreas), chymotrypsin (type II from bovine pancreas) and peptidases (grade III from hog intestinal mucosa) were all purchased from Sigma Chemical Company, St. Louis, MO. Casein was used as a reference protein. The in vitro digestibility data were subjected to analysis of variance and Duncan's multiple range test.

RESULTS & DISCUSSION

Extent of acylation

Table 1 shows the level of modification of oat protein as determined from the free lysine groups by DNBS method. Acetic anhydride seems to be more reactive than succinic anhydride. The protein contents of the unmodified and

Table 1—Extent of modification of oat proteins treated with acetic or succinic anhydride[a]

Anhydride	Anhydride conc. (g/g protein)	Available lysine (g/100g protein)	Extent of modification (%)
Control	0.00	3.54	0.0
Acetic	0.05	1.76	50.2
Acetic	0.20	0.50	86.1
Succinic	0.05	2.38	32.8
Succinic	0.20	1.35	61.9

[a] Average of duplicate determinations.

acylated oat preparations ranged between 62-67% (N X 6.25) and can be regarded as protein concentrates.

Amino acid composition

Table 2 lists the essential amino acid profiles of the unmodified and acylated oat proteins. Acylation caused a slight decrease in valine, isoleucine and phenylalanine, and consequently a lowering in the total essential amino acid content. The succinylated oat proteins had an essential amino acid profile similar to the control. Although lysine content did not change significantly in the acylated proteins, it has been demonstrated that acetyl- and succinyl-lysine derivatives were only partially utilized (Bjarnason and Carpenter, 1969; Mauron, 1972; Groninger and Miller, 1979).

Gel filtration chromatography

Fig. 1 shows the Sephacryl S-200 gel chromatograms of the unmodified (A), 61.9% succinylated (B), and 86.1% acetylated (C) oat proteins. The resolution of the protein peaks achieved with 2M sodium thiocyanate was much better than that with AUC (0.1M acetic acid, 3M urea, and 0.1M hexadecyltrimethyl ammonium bromide) buffer previously used (Ma, 1983a). The large peak at void volume represents high molecular weight aggregates (Ma, 1983a).

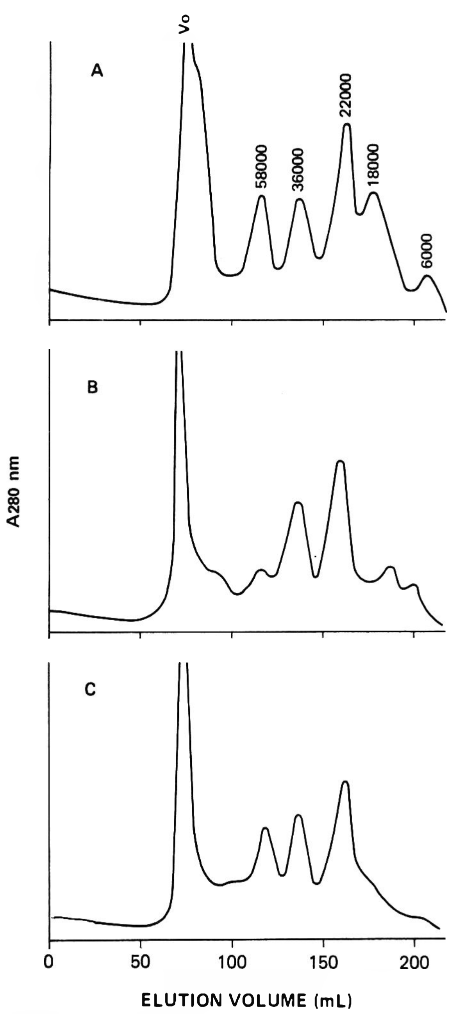

Fig. 1—Gel filtration chromatography of oat protein on Sephacryl S-200 column (2.5 X 45 cm), using 2M sodium thiocyanate as eluant. Flow rate was 25 mL/hr. The numbers above the peaks represent the estimated molecular weights of the fractions. Vo = void volume; A = unmodified oat protein; B = succinylated oat protein (61.9%); C = acetylated oat protein (86.1%).

The next three peaks with estimated MW of 58,000, 36,000, and 22,000 dalton correspond, respectively, to the dimer and the two monomers of oat globulins (Brinegar and Peterson, 1982), the major protein fraction in oats. The lower molecular weight components in the unmodified sample (Fig. 1A) could be derived from the minor protein fractions such as albumins and glutelins. Succinylation caused considerable dissociation of the void volume peak component and the globulin dimer, with corresponding increase in the two monomer fractions (Fig. 1B). Dissociation of protein complexes is common in succinylated samples and is caused by the introduction of repulsive forces between negative succinyl groups. Acetylation also caused some dissociation of the void volume component, but not the globulin dimer (Fig. 1C). The 18,000 and 6,000 dalton peaks were not

Table 2—Essential amino acid contents of acylated oat proteins (g/100g protein)[a]

Amino acid[b]	Control (0.0)[c]	Acetylated (50.2)[c]	Acetylated (86.1)[c]	Succinylated (32.8)[c]	Succinylated (61.9)[c]
Lysine	3.5	3.7	3.7	3.5	3.2
Threonine	3.0	3.2	3.2	3.1	3.6
Cystine	2.9	1.2	1.3	2.7	3.0
Methionine	1.4	1.5	1.5	1.6	1.5
Valine	5.1	4.2	4.0	5.1	5.2
Isoleucine	3.7	3.1	2.9	3.4	3.5
Leucine	7.5	7.4	7.2	7.5	7.6
Tyrosine	4.5	4.4	4.2	4.0	4.4
Phenylalanine	6.2	5.3	5.4	5.4	5.5
Total	37.8	34.0	33.4	36.3	37.5

[a] Average of duplicate determinations.
[b] Tryptophan was not determined.
[c] % modified.

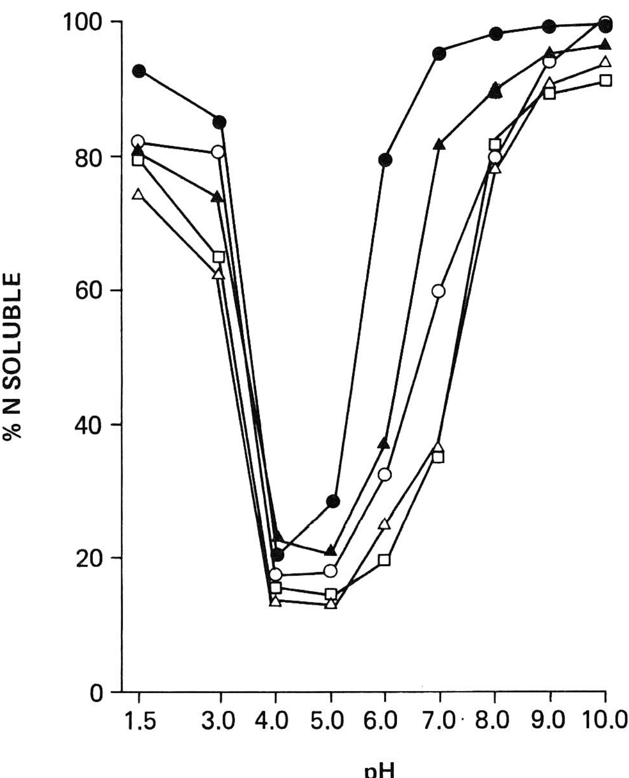

Fig. 2—Nitrogen solubility curves of acylated oat protein. □ = unmodified; ○ = succinylated (32.8%); ● = succinylated (61.9%); △ = acetylated (50.2%); ▲ = acetylated (86.1%).

resolved in the acetylated sample. The increase in net charge in these components may enhance interaction with the gel matrix and lead to poor resolution.

Functional properties

Solubility. Fig. 2 shows the solubility curves of the unmodified and acylated oat proteins. The familiar bell-shaped curve was observed for all the protein samples with minimum solubility between pH 4 and 5. Succinylation increased markedly the solubility of oat proteins at all pHs, especially at acidic and near neutral pH. Acetylation also improved solubility of oat protein, although the increase was not as pronounced as in the succinylated samples.

Emulsifying properties. The emulsifying capacity (EC), emulsifying activity index (EAI) and emulsion stability index (ESI) of the unmodified and acylated oat proteins are presented in Table 3. An improvement in both EC and EAI by acylation is evident, particularly with a higher degree of modification. The emulsion stability was greatly improved even at lower level of acylation.

The EC of meat-oat protein blends was also measured to determine the effect of acylation on meat-oat protein interaction. The results (Table 4) show that oat protein dispersions prepared in 1M NaCl had EC slightly lower than that in phosphate buffer (Table 3), suggesting an inhibitory effect of salt on emulsification. All the oat protein samples had EC higher than meat and the EC of the mixtures decreased progressively with increase in the ratio of meat in the blends. However, a 75:25 mixture of oat proteins:meat had EC close to or slightly higher than that of the oat proteins alone. The data suggest that interaction between oat and muscle proteins may have a synergistic effect on emulsification, and acylation seems to promote such effect.

Table 3—Emulsifying properties of acylated oat proteins[a]

Extent of acylation (%)	EC[b] (mL oil/g protein)	EAI[c] (m^2/g)	ESI[d] (min)
0.0	1993	40.4	6.2
Acetylated, 50.2	2116	40.8	13.0
Acetylated, 86.1	2280	43.6	16.5
Succinylated, 32.8	2132	44.0	19.2
Succinylated, 61.9	2352	56.2	21.0

[a] Average of three determinations.
[b] Emulsifying capacity.
[c] Emulsifying activity index.
[d] Emulsion stability index.

Table 4—Emulsifying capacity (EC) of meat-oat protein blends[a]

Extent of acylation %	EC (mL oil/g sample) 100:0[b]	75:25	50:50	25:75	0:100
0.0	1894	1829	1710	1440	1237
Acetylated, 50.2	1930	1998	1759	1564	1237
Acetylated, 86.1	2044	2015	1810	1580	1237
Succinylated, 32.8	1897	1949	1645	1536	1237
Succinylated, 61.9	2197	2283	2110	1533	1237

[a] Average of three determinations.
[b] Ratio of oat protein: meat.

Table 5—Water and fat binding properties of acylated oat proteins[a]

Extent of acylation (%)	Bulk density (g/mL)	WHC[b] (mL/g)	FBC[c] (mL/g)
0.0	0.31	2.00	2.10
Acetylated, 50.2	0.14	1.95	4.95
Acetylated, 86.1	0.11	1.35	6.35
Succinylated, 32.8	0.13	1.65	5.25
Succinylated, 61.9	0.10	1.45	6.30

[a] Average of duplicate determinations.
[b] Water hydration capacity.
[c] Fat binding capacity.

The EAI of the oat protein preparations was also determined at various pHs between 1.5 and 10.5. Fig. 3 shows that the pH-EAI curves of the oat protein samples resemble the pH-solubility curves (Fig. 2), with minimum emulsifying activity at pH 4.5. Acylation increased EAI at all pHs except pH 1.5, and the effect was more pronounced at pH between 3 and 6. Succinylated oat proteins had higher EAI than the acetylated samples, particularly at alkaline pH. The data indicate that while the improvement in emulsifying activity by acylation may not be highly significant at neutral pH, the increase could be substantial at slightly acidic or alkaline pH.

Water and fat binding properties. Table 5 lists the water hydration capacity (WHC) and fat binding capacity (FBC) of unmodified and acylated oat proteins. The bulk density was also determined and was found to decrease markedly in the acylated samples. This may contribute to the dramatic increase in FBC in the acylated proteins since fat absorption is partly related to the physical entrapment of oil by the protein matrix (Kinsella, 1976). In contrast, WHC was decreased by acylation, which could be attributed to the increase in protein solubility. It has been reported that highly soluble protein exhibits poor water absorption (Hermansson, 1973). The water-binding capacity of soy isolate was also decreased by acetylation, and was attributed to the elimination of the charged ϵ-amino groups of lysine (Barman et al., 1977).

Foaming properties. The foamability and foam stability of the unmodified and acylated oat proteins are shown in

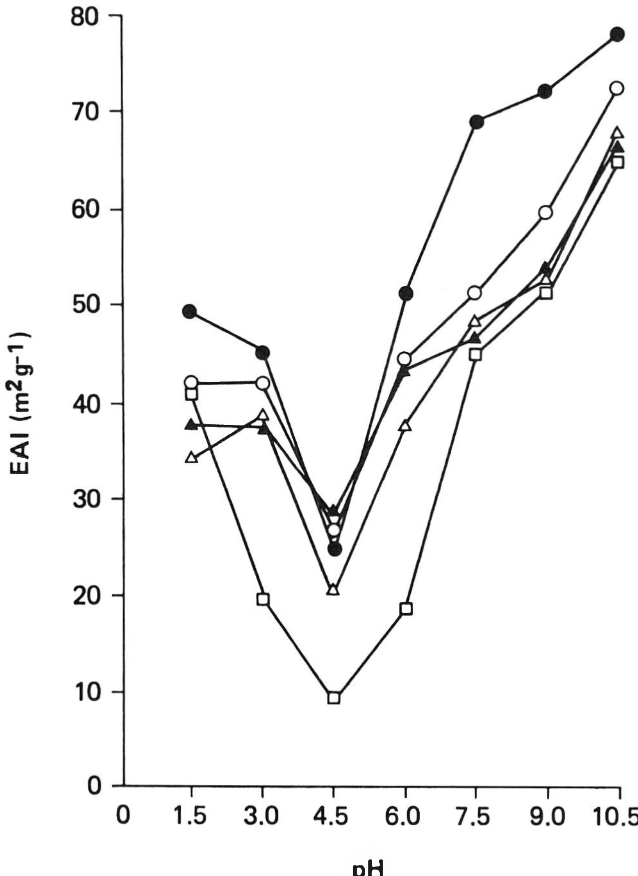

Fig. 3—EAI of acylated oat protein at various pH. □ = unmodified; ○ = succinylated (32.8%); ● = succinylated (61.9%); △ = acetylated (50.2%); ▲ = acetylated (86.1%).

Table 6—Foaming properties of acylated oat proteins[a]

Extent of acylation (%)	Foamability (%)	Foam stability (%)	
		30 min	60 min
0.0	85	70	53
Acetylated, 50.2	100	56	50
Acetylated, 86.1	90	42	35
Succinylated, 32.8	125	35	25
Succinylated, 61.9	95	30	22

[a] Average of duplicate determinations.

Table 7—In vitro digestibility of acylated oat proteins

Extent of acylation (%)	In vitro digestibility[a] (%)
0.0	82.27 ± 0.48[b]
Acetylated, 50.2	83.16 ± 1.22[b]
Acetylated, 86.1	85.95 ± 0.39[c]
Succinylated, 32.8	82.68 ± 0.70[b]
Succinylated, 61.9	85.86 ± 0.20[c]

[a] Mean ± S.D. of four samples.
[b,c] Means with the same superscript are not significantly different at 5% level by Duncan's Multiple Range Test.

Table 6. Foamability was increased in the acylated samples, but the foam stability was decreased by both acetylation and succinylation. Both foamability and foam stability decreased with increase in extent of modification. Although acylation increased protein solubility and soluble protein contributes to foaming, an excessive increase in charge may reduce the cohesive interactions between modified proteins and lower the surface film rigidity or even prevent the formation of elastic film at the air-liquid interface, hence reducing foamability and foam stability.

In vitro digestibility

Table 7 presents the in vitro digestibility of unmodified and acylated oat proteins measured by the multi-enzyme hydrolysis procedure (Hsu et al., 1977). The results show that the highly acylated protein had a significantly ($p < 0.05$) higher in vitro digestibility than the unmodified control. Modification at lower level did not alter the digestibility of oat protein. The improvement in digestibility may be due to an increase in solubility and the dissociation or unfolding of the protein molecules, making them more accessible to proteolytic enzymes. Using the same multi-enzyme assay, the digestibility of acylated pea protein was found to increase (Johnson and Brekke, 1983). However, a decrease in in vitro digestibility was reported in a number of succinylated proteins, particularly in the release of lysine (Groninger and Miller, 1979; Matoba and Doi, 1979; Siu and Thompson, 1982). A detailed evaluation of the digestibility and nutritional quality of acylated oat protein requires more elaborate in vivo tests.

CONCLUSION

THE PRESENT DATA show that acylation improved some functional properties of oat protein, including solubility, emulsifying properties and fat-binding capacity. The results of the meat-oat protein emulsion blends suggest favorable interaction between acylated oat proteins and muscle proteins. Most of these changes were more pronounced with succinylated protein than acetylated protein. As in other acylated plant proteins, the improvement in functionality can be attributed to changes in the physicochemical characteristics of the proteins resulting from altered conformation and an increase in net negative charge. The functionality improvement will enhance the value of oat protein and make it more competitive with other widely used food proteins. The acylated oat protein, with excellent emulsifying and fat binding properties, should find application in many fabricated food systems such as meat emulsion products and salad dressing.

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Bioavailability of Zinc and Iron from Mature Winged Bean Seed Flour

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ABSTRACT

The relative bioavailability of zinc and iron from mature winged bean (*Psophocarpus tetragonolobus*) flour was determined utilizing standard rat bioassay procedures. Weight gain of rats after 21 days and total bone (tibia) zinc resulting from zinc addition to the standard diet as zinc carbonate or from zinc endogenous to winged bean flour were compared. The relative bioavailability of winged bean zinc was calculated to be 85% ($P < 0.05$) and 93% (N.S.) when weight gain and log tibia zinc were the criteria of evaluation. The results of the hemoglobin repletion assay indicated that iron from winged bean was 89% (N.S.) as bioavailable iron from ferrous sulfate.

INTRODUCTION

WHILE A CONSIDERABLE NUMBER of published reports have dealt with the proximate composition, amino acid and fatty acid profiles of various cultivars of mature winged bean flour (Agcaoili, 1929; Claydon, 1975; Watson, 1977; Ekpenyong and Borchers, 1980; Garcia and Palmer, 1980), few have dealt with the micronutrient composition (Cerny, 1980; Okezie and Martin, 1980). None has investigated the bioavailability of minerals from this legume (International Grain Legume Centre, 1978; Aterrado, 1979). The winged bean *Psophocarpus tetragonolobus* shows considerable promise as a high protein, high energy food source for inhabitants of the humid tropics (NAS, 1981).

Evaluation of the amount of utilizable nutrients, such as zinc and iron, from mature seeds is important in light of their marginal consumption status in many areas throughout the world. The objective of this work was to determine the relative bioavailability of zinc and iron from mature winged bean flour using standard rat bioassay procedures.

MATERIALS & METHODS

Preparation of winged bean flour

Zinc study. Mixed whole winged beans (TPT_2, Chimbu, SLS_1; ratio 2:1.5:1.0, obtained from Peradeniya, Sri Lanka) were cleaned and heated for 30 min at 93°C in a Proctor Standard Variable Circulator Drier (Proctor and Schwartz, Inc., Philadelphia, PA). the hot beans were partially cracked by passing them through a spinning drum plate dehuller and were soaked overnight (16 hr) at 2°C in 3 parts cold distilled water (weight/weight).

Subsequently the beans were blanched for 40 min in 5 parts boiling deionized water (weight/weight). After draining and rinsing the beans with deionized water, the hulls were removed by hand. The cotyledons were ground with an equal weight of deionized water using a Rietz Disintegrator (Rietz Manufacturing Company, Westchester, PA) operated at a rotor speed of 10,000 rpm and fitted with a 0.023 inch screen. The slurry was reground and then drum dried on a double-drum drier (Mathis Co., South Bend, IN). The dryer was operated at 2.81 kg/cm^2 gauge. The space between the hot drums was set at 0.25 mm. The dried winged bean flour flake was passed through a 40 mesh screen with gentle rubbing and stored at 2°C until use.

Iron study. Mixed variety beans (Chimbu, TPT_2, SLS_{20}; ratio 2:1:1) were cleaned and soaked in deionized water (seeds to water 1:10 wt/vol) for 16 hr at 4°C and dehulled manually. The cotyledons were autoclaved for 20 min at 1.05 kg/cm^2 gauge (120°C) and dried at 60°C in a hot air oven for 18 hr. The dried cotyledons were ground in a Waring Blendor to pass through a 40 mesh sieve. The resultant flour was stored at 2°C until use.

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Analysis of flours, diets and bones

Proximate analysis of samples were determined by following the standard AOAC (1975) procedures: crude protein by Kjeldahl (N X 6.25, section 2.049); crude fat by ether extraction (section 14.080) and moisture content by weight difference (section 14.076). Selected minerals were analyzed by atomic absorption spectrophotometric analysis (Perkin-Elmer model, 306 Norwalk, CN) of (a) wet ashed samples (nitric acid digestion of winged bean flour and diets, Forbes and Parker, 1977) and (b) dry ashed bone samples (650°C for 32 hr). Phosphorus and phytic acid were analyzed by the method of Bartlett (1959) and the supernatant difference method of Thompson and Erdman (1982), respectively. Proximate composition of the mature winged bean flour used for zinc and iron studies is shown (Table 1).

General procedures for bioavailability studies

Zinc study. The zinc bioassay followed procedures previously reported from our laboratory (Forbes et al., 1979; Erdman et al., 1980). Sixty-six male weanling Sprague-Dawley rats (initial average body weights of 57g) were obtained from Harlan Industries, Inc. (Cumberland, IN). The rats were housed in individual stainless steel cages and fed with standard lab chow pellets (Ralston Purina Co., St. Louis) for a 2-day adaptation period. The room was maintained between 21-22°C and regulated to 12 hr light – 12 hr dark schedule in which the light period was initiated at 0600 hr. After the adaptation period, the rats were weighed and assigned to one of 11 experimental groups. Rats were fed these diets and deionized water *ad libitum* for 21 days. Disks were cut out of plastic bottles and placed between the water bottle and cage to prevent the rats from chewing on the rubber stoppers. Feed intake and weight gains were monitored.

All test diets were made up on an isoenergetic and isonitrogenous (20% protein) basis. The general make-up of the egg white and the winged bean test diets is given in Table 2. The control egg white diets were supplemented with 0, 2, 5, 7, 10, or 50 ppm zinc as $ZnCO_3$. The bioavailability of zinc in winged bean flour was tested by substituting winged bean flour for egg white on an equivalent protein basis to provide 2, 5, 7, or 10 ppm zinc from the winged bean flour. As an example, the 2 ppm winged bean diet contained 4.5% winged bean and 22.74% egg white. When combined in this manner, the diet supplied 20% protein and 8% fat, the same as the

Table 1—Composition of winged bean flour[a]

	Zinc study	Iron study
Moisture (%)	4.5	5.0
Protein (%)	40.2	41.6
Fat (%)	21.0	19.8
Calcium (%)	0.41	0.48
Phosphorus (%)	0.46	0.48
Magnesium (%)	0.22	0.23
Zinc (ppm)	44.4	47.5
Iron (ppm)	75.5	83.6
Copper (ppm)	20.0	26.6
Phytic Acid (%)[b]	1.36	1.04

[a] Expressed as "as is" basis. Averages of duplicate analysis.
[b] Assuming 23.2% phosphorus in phytic acid.

control diet. A 50 ppm winged bean diet was made by adding 40 ppm zinc as $ZnCO_3$ to the 10 ppm winged bean diet. Since sulphur-containing amino acids are limiting in winged bean protein, 0.3% L-methionine was added to winged bean flour diets to help equilibrate protein quality. Adjustments were made in corn oil, $CaCO_3$, and K_2HPO_4 to make diets isocaloric and of equal calcium and phosphorus content. At the end of the test feeding period, rats were weighed, killed with ether and both tibias were removed. The bones were placed in boiling deionized water for 1 min and the residual flesh removed. The bones were dried at 95°C overnight. Subsequently, the bones were dry ashed at 650°C for 32 hr and then dissolved in dilute HCl (10:1 deionized water:HCl; vol:vol).

Iron studies. Iron bioavailability from winged bean flour was evaluated by a hemoglobin repletion assay procedure (Fritz et al., 1975). Sixty male Sprague-Dawley rats weighing about 65g were individually housed in stainless steel cages in a controlled temperature environment for 6 wk. The rats were fed a low iron depletion diet (see Table 3) containing 4.51 ppm iron for 4 wk to reduce their body stores of iron. After 4 wk the average blood hemoglobin levels had fallen to 6.29 g/100 mL (range 4.65-7.20). Hemoglobin (Hb) content of blood was determined by the cyanmethemoglobin reaction (Hycel #116C, Hycel Inc., Houston, TX). Blood was obtained from the tail vein after the 4-wk depletion period and after the 2-wk repletion period. The rats were divided into groups of eight or nine rats each. Each group had similar average Hb and average body weight (216g). During the 2 wk regeneration period, seven different diets were fed and relative iron bioavailability was determined by Hb gain (Fritz et al., 1975) as well as by total body Hb gain per mg iron intake.

Control diets (Table 3) were casein-based with 0, 6, 12, or 18 ppm iron added as ferrous sulfate. Experimental winged bean diets were formulated to provide 6, 12, or 18 ppm iron from the winged bean. All diets were designed to be isocaloric and isonitrogenous. Adjustments in corn oil and casein were made to achieve the desired fat and protein levels in the diets.

Statistical analysis

The data for zinc studies and for the hemoglobin repletion assay were statistically analyzed by regression analysis. Then, the slopes of the curves for weight gain or log tibia zinc (for zinc study) or gain in hemoglobin levels (iron study) versus mineral level in the diet were compared for the different mineral sources (Momcilovic et al., 1975; Fritz et al., 1975; Steel and Torrie, 1960). Relative slope ratios were calculated by comparing the slopes of the linear regression lines. In addition, standard analysis of variance and least significant difference tests were performed on data derived at each dietary iron level (Steel and Torrie, 1960).

RESULTS & DISCUSSION

TABLE 1 LISTS the proximate composition of the winged bean. The phytic acid content of the winged bean flour for

Table 2—Composition of basal egg white and winged bean flour diets for zinc study

Ingredient	Egg white basal diet (%)[a]	Winged bean diets (%)[a]
Egg white[b]	25.0	13.6 – 22.74
Winged bean flour[c]	—	4.5 – 22.52
Corn oil[d]	8.0	3.27 – 7.05
Zn-free salts[e]	3.33	3.33
Fiber[f]	3.0	3.0
Vitamin mix[g]	1.0	1.0
NaCl	0.0071	0.0021 – 0.0057
$CaCO_3$	0.2299	0.0689 – 0.1839
KCL	0.1483	0.0444 – 0.1187
K_2HPO_4	0.1519	0.0455 – 0.1216
Glucose	59.13	53.0 – 57.65

[a] Expressed on "as is" basis.
[b] Purchased from Semour Foods (Topeka, KS).
[c] Prepared as described under Materials & Methods.
[d] Purchased from Teklad Test Diets (Madison, WI).
[e] Contained in g/kg mix: NaCl, 130.5; K_2CO_3, 95.3; K_2SO_4, 54.2; K_2HPO_4, 92.6; $CaHPO_4$, 337.5; $CaCO_3$, 202.2; $MgCO_3$, 64.1; $FeC_6H_5O_6 \cdot H_2O$ (16% Fe) 19.2; $MnSO_4 \cdot H_2O$, 4.5; $CuSO_4 \cdot 5H_2O$, 0.65; KIO_3, 0.20; Na_2SeO_3, 0.06.
[f] Solka Floc, Brown Co. (Berlin, NH).
[g] Teklad #40060 with biotin added to provide 4.44 mg/kg diet; p-amino benzoic acid, 110.1; ascorbic acid 1016.6; B_{12}, 29.7; calcium pantothenate, 66.7; choline chiliydrodihydrogen citrate, 3496.9; folic acid, 2.0; l-ionositol, 110.1; menadione, 49.6; nicotinic acid, 99.1; pyridoxine HCl, 22.0; riboflavin, 22.0; thiamin HCl, 22.0; units/kg dry diet, retinyl acetate, dry (5947.2 R.E.); ergocalciferol, (dry) 2202.5; tocopherol acetate, 121.2 IU.

Table 3—Composition of iron depletion, control and test diets[a]

Ingredient	Depletion diet[b,c] (%)	Control diet (%)	Test diets (%)
Winged bean flour	—	—	7.18 – 21.53
Casein, vitamin-free test[d]	20	20	9.37 – 16.45
Corn oil[d]	5.0	5.0	0.74 – 3.58
Mono sodium phosphate	2.0	2.0	2.0
$CaCO_3$	2.0	2.0	2.0
KCl	0.5	0.5	0.5
Iodized salt	0.5	0.5	0.5
Fe-free trace mineral mix[e]	0.27	0.27	0.27
Vitamin pre-mix[d,f]	1.0	1.0	1.0
D.L. methionine[g]	0.1	0.1	0.1
Cerelose[h]	68.63	68.63	61.99 – 66.42
Added Fe[i]	0.0	variable	variable

[a] Expressed on "as is" basis.
[b] Fritz et al. (1975).
[c] Iron content upon analysis, 4.5 ppm.
[d] Purchased from Teklad Test Diets (Madison, WI).
[e] Fritz et al. (1974).
[f] See footnote g, Table 2
[g] Ajinomoto Co., Inc., (Tokyo).
[h] Added to bring diets up to 100%.
[i] 0, 6, 12, or 18 ppm Fe from ferrous sulfate or winged bean flour.

Table 4—Weight gain, feed efficiency, total zinc consumed and tibia zinc of rats fed egg white or winged bean-based diets[a]

Diet	Zinc (ppm) (Calculated)	Zinc (ppm) (Actual)[b]	Weight gain (g)	Feed efficiency[c]	Zinc consumed (mg) (actual)	Tibia zinc[d] μg Zn	Tibia zinc[d] Log μg Zn
Egg White	0	1.4	13 ± 5	0.08 ± 0.03	0.17 ± 0.01	21.3 ± 0.35	1.34 ± 0.01
	2	2.5	30 ± 5	0.15 ± 0.02	0.61 ± 0.04	28.6 ± 0.61	1.45 ± 0.02
	5	5.3	68 ± 7	0.29 ± 0.04	1.31 ± 0.08	33.8 ± 0.92	1.53 ± 0.03
	7	7.9	95 ± 7	0.37 ± 0.04	1.96 ± 0.06	40.9 ± 0.53	1.61 ± 0.02
	10	10.3	114 ± 17	0.39 ± 0.05	3.29 ± 0.16	63.3 ± 1.40	1.80 ± 0.02
	50	50.7	127 ± 14	0.44 ± 0.04	14.85 ± 0.88	136.2 ± 2.00	2.13 ± 0.02
Winged Bean	2	2.3	23 ± 8	0.11 ± 0.04	0.58 ± 0.05	27.2 ± 1.05	1.43 ± 0.04
	5	5.0	56 ± 8	0.24 ± 0.05	1.39 ± 0.14	33.1 ± 1.03	1.52 ± 0.03
	7	7.5	76 ± 12	0.30 ± 0.06	1.91 ± 0.17	36.9 ± 0.88	1.57 ± 0.01
	10	9.0	99 ± 5	0.33 ± 0.04	3.37 ± 0.29	58.8 ± 0.83	1.77 ± 0.01
	50	50.1	114 ± 9	0.38 ± 0.03	14.67 ± 0.82	130.0 ± 2.25	2.12 ± 0.02

[a] Expressed as group means ± S.D. for 21 days of feeding (N=6).
[b] Mean of three determinations.
[c] Weight gain (g) ÷ food intake (g) for 21 days.
[d] Total zinc for two tibias.

Table 5—Statistical analysis of results from Table 4

Diets	r	Regression equations	Slope ratio	t' between slopes
		(A) Weight gain of rats vs dietary zinc concentration		
Egg	0.97	Yw = 10.59 X + 12.73[a]	0.85	2.39
Winged bean	0.97	Yw = 9.01 X + 10.04		($P < 0.05$)
		(B) Weight gain of rats vs total actual zinc consumed		
Egg	0.94	Yw = 33.18 X + 14.82[b]	0.83	1.97
Winged bean	0.94	Yw = 27.40 X + 12.55		(N.S.)
		(C) Log μg tibia zinc vs dietary zinc concentration (ppm)		
Egg	0.98	Yt = 0.044 X + 1.135[c]	0.93	1.09
Winged bean	0.97	Yt = 0.041 X + 1.328		(N.S.)
		(D) Log μg tibia zinc vs total actual zinc consumed		
Egg	0.98	Yt = 0.141 X + 1.34[d]	0.86	2.18
Winged bean	0.96	Yt = 0.121 X + 1.34		($P < 0.05$)

[a] Yw = body weight gain (g) for 21 days; X = dietary zinc concentration (ppm).
[b] Yw = body weight gain (g) for 21 days; X = actual (by analysis) total zinc consumed (mg) for 21 days.
[c] Yt = log μg zinc per two tibia; X = dietary zinc concentration (ppm).
[d] Yt = log μg zinc per two tibia, X = actual (by analysis) total zinc consumed (mg) for 21 days.

Table 6—Iron content of diets for iron study

Diets	Iron content (ppm) Calculated	Actual[a]
Casein	0	4.7
	6	7.9
	12	11.5
	18	18.0
Winged Bean	6	7.5
	12	12.1
	18	18.6

[a] Mean of three determinations.

Table 7—Mean weight gain, feed intake and feed efficiency of iron depleted rats fed test diets during the depletion period[a]

Diet group	Added dietary iron level (ppm) 0	6	12	18
	(A) 2-wk Gain (g)			
Casein	26 ± 4	53 ± 6	66 ± 6	78 ± 4
Winged bean	—	50 ± 7	60 ± 7	78 ± 5
	(B) 2-wk Feed intake (g)			
Casein	250 ± 19	258 ± 16	276 ± 8	295 ± 13
Winged bean	—	260 ± 13	273 ± 8	283 ± 13
	(C) Feed Efficiency (g gain/g feed intake)			
Casein	0.01 ± 0.01	0.20 ± 0.02	0.23 ± 0.01	0.26 ± 0.01
Winged bean	—	0.21 ± 0.02	0.26 ± 0.02	0.27 ± 0.02

[a] Rats fed an iron depletion diet for 4 wk were switched to test diets and maintained on these diets ad libitum for 2 wk. Data points represent mean ± S.D. (N = 8 or 9). Statistical analysis of diet groups at single dietary iron levels revealed no difference in gain, intake or feed efficiency due to diet type at any iron intake level ($P < 0.05$).

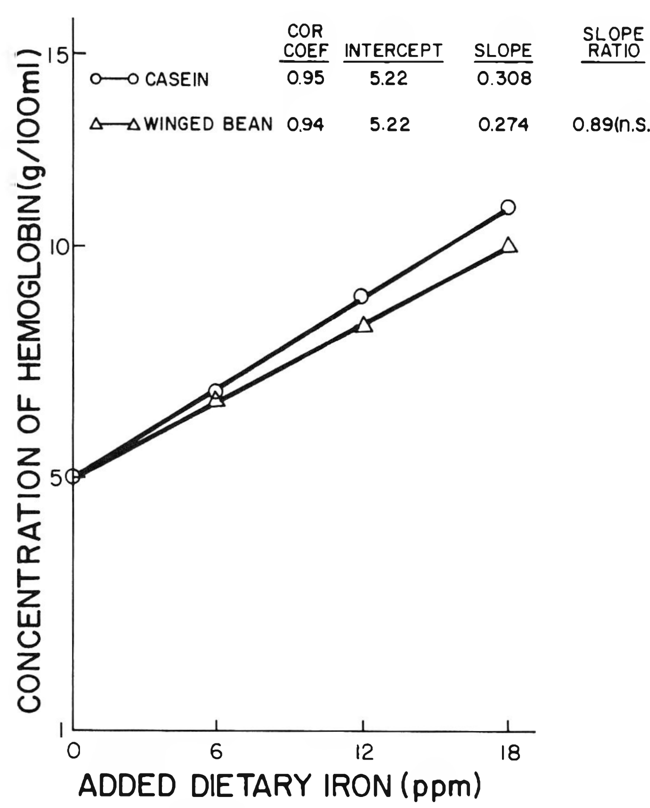

Fig. 1—Final hemoglobin concentration of iron depleted rats fed repletion diets.

the iron study was lower than that for the zinc study. This difference can be attributed to one or more of the following: (a) different proportion and type of cultivars used to prepare the flours, (b) difference in processing methods, or (c) longer storage of winged bean flour for the iron study. The flour utilized for the iron study was processed and stored, tightly sealed at 2°C for several months prior to the animal experiment. Unpublished data from our laboratory showed that cotyledons from 14 winged bean cultivars contained from 1.03–1.67% (d.b.) phytic acid with a mean vaue of 1.42%. Other than phytic acid, the composition of the two flours was quite similar.

Zinc study

The zinc content (calculated and analyzed) of the egg and winged bean-based diets, total zinc consumed during the test period, as well as the results of the feeding studies are given (Table 4). Food consumption of rats fed the same zinc levels were similar for each protein source. Statistical analysis of weight gain and log tibia zinc data are reported (Table 5). The results were calculated both on the basis of zinc concentration in the diets and upon total zinc (by analysis of diets) consumed over the experimental period.

Comparison of the slopes of the linear regression lines show that the zinc from winged bean flour was utilized

Table 8—Mean hemoglobin concentration, total body hemoglobin gain, iron intake and total body hemoglobin gain per mg iron intake of iron depleted rats fed repletion test diets[a]

Diet group	Added dietary iron level (ppm) 0	6	12	18
	(A) Final Hb concentration (g/100 mL)			
Casein	5.2 ± 0.7	7.0 ± 0.4	9.0 ± 0.7	10.7 ± 1.0
Winged bean	—	6.7 ± 0.4	8.8 ± 0.6	10.0 ± 1.0
	(B) Total body Hb gain (g)[b,c]			
Casein	−0.08 ± 0.05	0.34 ± 0.08	0.77 ± 0.08	1.13 ± 0.13
Winged bean	—	0.30 ± 0.09	0.72 ± 0.12	1.04 ± 0.15
	(C) Total iron intake (mg)[c]			
Casein	1.18 ± 0.09	2.05 ± 0.14	3.15 ± 0.09	5.31 ± 0.24
Winged bean	—	1.95 ± 0.09	3.30 ± 0.09	5.20 ± 0.22
	(D) Total body Hb gain (g)/mg iron intake[c]			
Casein	−0.07 ± 0.04	0.17 ± 0.04	0.24 ± 0.03	0.21 ± 0.02
Winged bean	—	0.15 ± 0.04	0.22 ± 0.03	0.19 ± 0.02

[a] The treatment of rats is described in footnote a of Table 7. No significant differences were noted for comparisons at single dietary iron level ($P < 0.05$).
[b] Calculated assuming blood volume to be 5.46% of body weight of rat (Lombardi and Oler. Lab. Invest. 17:308, 1967).
[c] Calculated during 2-wk repletion period only.

85% ($P < 0.05$) and 83% (N.S.) as efficiently as zinc from zinc carbonate added to egg white-based diets for weight gain when expressed as zinc concentration of diet and total zinc consumed, respectively (Table 5A and 5B). These results show that zinc was well utilized for growth of the rat. The log μg tibia zinc data indicate that zinc from winged bean also was well utilized for bone mineralization compared with a good source of zinc, zinc carbonate (Table 5C and 5D).

The results from the zinc bioassay can be compared to previous results for various soybean products. Erdman and Forbes (1981) summarized the zinc availability of several soy products and showed that the availability of zinc in soy foods depend upon the phytate to zinc molar ratio as well as the processing methods employed. Full fat soy flour, which has almost identical zinc, phytic acid and proximate composition as the winged bean, was utilized 55% and 34%, as well as zinc carbonate for growth and bone mineralization, respectively. Therefore, full fat winged bean flour would appear to be a good plant source for zinc.

Iron study

The calculated and actual concentration of iron in the casein and winged bean diets are shown (Table 6). Other than in the 0 iron casein diet, the actual concentration was quite close to the calculated values.

The final hemoglobin concentration of groups of rats during a 2-wk repletion period can be plotted (Fig. 1). The results from this standard hemoglobin repletion test indicate that winged bean iron was well utilized by rats (89% as available as ferrous sulfate added to a casein-based diet). It should be noted that the standard vitamin mix, and thus all casein and winged bean diets, used for this study did contain ascorbic acid, which is well known to enhance nonheme iron absorption (Lynch and Cook, 1980). However, control diets derived the same enhancement as did winged bean diets.

The weight gain, feed intake, feed efficiency, final Hb concentration, total body Hb gain, iron intake and the total body Hb gain per mg iron intake for groups of rats over the 2-wk repletion period are shown in Tables 7 and 8. Statistical analysis of diet groups at single dietary iron levels revealed no differences in any measured parameters due to diet type at any iron intake level ($P < 0.05$).

The results from the iron bioassay suggests that iron from winged bean is well utilized by the rat. Another study from our laboratory (Picciano et al., manuscript submitted for publication) found that the bioavailability of iron from a commercial soy concentrate, a full fat soy flour and a soy beverage ranged from 66–92% of ferrous sulfate utilizing the same hemoglobin repletion assay (and the same vitamin mix).

We can conclude that both zinc and iron are well utilized by the rat from mature winged bean flour. It can be considered a good plant source for these trace minerals.

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Fate of Chlorine During Flour Chlorination

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ABSTRACT

Unbleached flour (pH 5.8) was treated with $^{36}Cl_2$ (g) in a closed-system flour chlorinator to pH levels of 5.25, 4.41, and 4.01. Of the total active Cl_2 generated (12.38, 20.71 and 28.17 mg, respectively), 98.6-99.6% was shown to react with the flour. Only 0.35-1.37% was recovered in NaOH traps. Extraction of the treated flour with chloroform showed that 27.0-33.7% of the total ^{36}Cl was incorporated into flour lipids, of which 31.2-39.1% of the radioactivity went to nonpolar lipids and 60.9-68.8% to polar lipids. The water-insoluble and water-soluble fractions of defatted flour contained 17.9-21.4% and 2.1-4.7% of the total ^{36}Cl, respectively. The remaining ^{36}Cl (40.3-49.1%) was present as $^{36}Cl^-$.

INTRODUCTION

TREATMENT OF CAKE FLOURS with chlorine gas has been practiced for many years as a means of improving baking quality (Kissell and Yamazaki, 1979; Wilson et al., 1964). Although many studies have been conducted to define the functional effects of flour lipids on cake-baking potential (Spies and Kirleis, 1978; Johnson et al., 1979; Kissell et al., 1979), the fate of chlorine during flour chlorination has not been well studied. Sollars (1961) studied the chlorine content of cake flours and flour fractions by combustion analysis in which all forms of chlorine were measured as chloride. Gilles et al. (1964) also analyzed chlorine in chlorine-treated flour and its fractions by X-ray spectrographic methods. Again, the methods employed were for "Cl" analysis and did not provide information as to the form of chlorine (i.e., active chlorine, incorporated chlorine, or chloride ion) in the treated flour.

In this study, the distribution of chlorine in chlorinated flour was studied by using radioactive chlorine gas to bleach flour. The chlorine content was determined as active chlorine, incorporated chlorine or chloride ion. This study was designed to determine: (a) the proportion of the Cl_2 used to bleach flour, which reacted with flour components, and (b) the percent distribution of reacted chlorine in the organic molecules of the flour, including the flour lipids, and the water-soluble and water-insoluble fractions of the defatted flour.

MATERIALS & METHODS

Samples

Unchlorinated soft flour (pH 5.80) was supplied by W. T. Yamazaki of the Soft Wheat Quality Laboratory, Ohio Agricultural Research & Development Center (Wooster, OH). It was 50% patent extraction grade and was further pin milled at 9000 rpm. This flour contained 7.4% protein (N x 5.7 at 14% m.b.), 0.28% ash, and 13.2% moisture. A commercial unbleached flour (Gold Medal Unbleached, Enriched, All Purpose, Pre-sifted Flour, General Mills, Inc., Minneapolis, MN) was purchased locally and used for comparison. It contained 9.7% protein, 76.7% carbohydrate, and had a pH of 6.2.

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Reagents

[^{36}Cl]-Hydrochloric acid was purchased from New England Nuclear (Boston, MA). All other chemicals used in this study were reagent or analytical grade.

pH Measurement of the flours

The pH of the unchlorinated and chlorinated flours was determined in test tubes following the modified procedures of the AACC Method 02-52 (Approved Methods of AACC, 1976). Ten mL cool (25°C) and recently boiled water were added to 1g flour.

Flour chlorinator and flour chlorination

The flour chlorinator (Fig. 1) used in this study was a closed system. It consisted of three main components, i.e., the chlorine gas generator (Burette A and Flask B), the flour reactor (Flask F), and the chlorine gas trap for unreacted Cl_2 (Flasks G and H). Chlorine was generated in Flask B following the method described by Ghanbari et al. (1983a). When 1 mL 6N H_2SO_4 was added dropwise from Burette A to Flask B, which contained 0.5g solid $KMnO_4$ and 100, 144 or 200 μL 4.62M $Na^{36}Cl$ solution ($H^{36}Cl$ was first neutralized with NaOH to $Na^{36}Cl$), enough $^{36}Cl_2$ was produced in Flask B, moved to Flask F and reacted with 20g flour (pH 5.80) and to change the pH of the flour to 5.25, 4.41 or 4.01, respectively (Kissell et al., 1979). During the chlorination process, the flour was agitated constantly to assure uniform chlorination. At this time, valves C_2 and C_4 were closed. When $^{36}Cl_2$ was no longer being produced and bubbling in Flask B stopped, valve C_1 was closed. The flour was exposed, with agitation, to $^{36}Cl_2$ for 5 more minutes and then valve C_2 was opened to allow any residual $^{36}Cl_2$ for 5 more minutes and then valve C_2 was opened to allow any residual $^{36}Cl_2$ to be trapped in NaOH solutions in Flasks G and H. With the system under vacuum, valve C_4 was opened for 15-20 min to remove and trap any residual $^{36}Cl_2$ in Flasks G and H. Flask B was then removed from the system and valve C_1 was opened to sweep any $^{36}Cl_2$ in the headspace of Flask F into the NaOH traps. The treated flour in Flask F was stored at 5°C for further analysis. The NaOH solutions in Flasks G and H were analyzed for available chlorine and radioactivity. The results reported herein represented means of duplicated studies.

Determination of available chlorine

Available chlorine was measured by iodometric titration (Franson, 1980; Masschelein, 1979).

Radioactivity measurement

Radioactivity was determined by scintillation counting of the samples dissolved in 15 mL Aquasol (New England Nuclear) using a Searle Analytic 92 Liquid Scintillation System with windows set at 50-800. Counting efficiency was determined by constructing a quench curve, and counter reliability was monitored by including a standard with each run. Flour samples (50-100 mg) were prepared for counting by dissolving in 0.25-0.5 mL of tissue solubilizer (ICN 8 02395) for 2 hr at 70°C, or overnight at ambient temperature.

Lipid extraction

Flour lipids from 20g chlorinated flour were washed at room temperature for 4 hr in a flask with chloroform (10 mL/g flour). After filtration through a Whatman No. 1 filter paper, the flour was resuspended twice in 100 mL chloroform and filtered. The combined

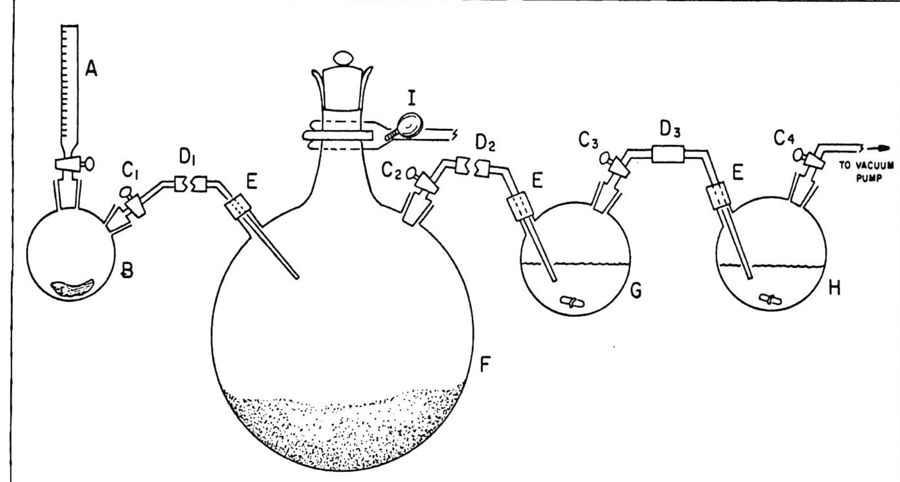

Fig. 1—Flour chlorinator. Where: (A) is Burette containing 6N H_2SO_4 (10 mL w 14/20 neck, Bantam-Ware, Kontes K299000-0010); (B) Two-neck boiling flask containing $KMnO_4$ and NaCl/$Na^{36}Cl$ (Kontes K294990-0025); (C) Flow control adapter (Kontes 275200); (D) Tygon tubing, lengths adjusted to provide flexibility for shaking of F flask; (E) Inlet tube (Kontes K-275501) and rubber adapter (Kontes K773900); (F) Three-neck boiling flask containing flour, a modified 500 mL round-bottom flask; (G) Two-neck boiling flask containing 20 mL 0.1N NaOH (Kontes 294990-0050); (H) Two-neck boiling flask (Kontes 294990-0050) containing 20 mL 1.0N NaOH; and (I) Shaker arm.

Table 1—Distribution of (^{36}Cl) radioactivity in the flour chlorinator after completion of the chlorination process. (20g of unbleached flour, pH 5.80, were chlorinated to pH 5.25, 4.41 and 4.01, using $^{36}Cl_2$ with a specific activity of 1.26 X 10^6 dpm/mg)

Flour pH	Total (^{36}Cl) used for chlorination	Total radioactivity in					
		Chlorinated flour		Trap 1		Trap 2	
	dpm X 10^7	dpm X 10^7	Percent[a]	dpm X 10^5	Percent[a]	dpm	Percent[a]
5.25	1.56	1.55	99.35	1.25	0.80	negligible	negligible
4.41	2.61	2.59	99.23	1.96	0.75	negligible	negligible
4.01	3.55	3.54	99.72	1.15	0.32	negligible	negligible

[a] These values are calculated by dividing the total radioactivity in flasks F, G and H to total (^{36}Cl) radioactivity used for chlorination.

chloroform washes were reduced to less than 10 mL in a rotory evaporator at 30°C, transferred to a 50 mL glass stoppered centrifuge tube and washed twice with an equal volume of water-methanol solution (2:1) to remove any $^{36}Cl^-$ which was associated with the lipid extract. The chloroform layer was removed and evaporated to dryness under a stream of N_2. The remaining lipid (total chloroform-soluble lipid) was redissolved in chloroform. Components of the total chloroform-soluble lipid were separated by liquid chromatography on silica gel 60 (70-230 mesh) columns and by thin layer chromatography according to the procedure described by Johnston et al. (1983).

Fractionation of defatted flour

The defatted flours were suspended in distilled water at a ratio of 10:1 (v/w H_2O:flour), and the pH of the suspension was adjusted to approximately 6 with 0.5 N NaOH. After stainding at room temperature for 4 hr, the suspension was centrifuged at 10,000 x *g* for 10 min. Aliquots of the supernatant, which contained water-soluble components, were analyzed for radioactivity. The chloride ion content and the extent of chlorine incorporation into water-soluble organic compounds in the supernatant were determined by the method of Ghanbari et al. (1982a).

The centrifugate (water-insoluble fraction) was washed with 10 mL 0.01M NaCl and centrifuged. This washing procedure was to minimize any nonspecific binding of $^{36}Cl^-$ by isotopic dilution and was repeated once. This water-insoluble fraction was then analyzed for radioactivity after an aliquot of the sample was dissolved in tissue solubilizer.

Experimental controls

To correct for nonspecific binding of $^{36}Cl^-$ in chlorine incorporation measurements, $Na^{36}Cl$ solution containing ^{36}Cl in an amount equivalent to the experimental sample, was added to untreated flour during chloroform extraction.

Commercial flour

A commercial unbleached flour (pH 6.2) was also chlorinated to pH 4.0 and subjected to similar experimental procedures. The data are reported and discussed at the end of each section of the results for comparison purposes.

Table 2—Fate of available chlorine during flour chlorination (Experimental conditions as in Table 1)

Flour pH	Total Cl_2 used for chlorination	Available chlorine[b] in			
		Flask F	Trap 1		Trap 2
	(mg)[a]	(mg)	mg	Percent[c]	(mg)
5.25	12.38	ND[d]	0.17	1.37	ND[c]
4.41	20.71	ND	0.17	0.82	ND
4.01	28.17	ND	0.10	0.35	ND

[a] Total available chlorine (Cl_2) was calculated by dividing the total radioactivity used to the specific activity of $^{36}Cl_2$ as listed in Table 1.
[b] Measured by idotitrometric assay.
[c] Percent of total Cl_2 used for chlorination.
[d] ND = not detectable.

RESULTS & DISCUSSION

Distribution of (^{36}Cl) radioactivity after chlorination process

The distribution of radioactivity in the flour chlorinator after the completion of flour chlorination process is reported in Table 1. The chlorinated flour in Flask F was found to contain more than 99% of the total radioactivity at each flour chlorination pH level. Flask G, the trap, contained only 0.32-0.80% of the total radioactivity; the percentage of radioactivity decreased as the acidity of the flour increased. Only negligible amounts of radioactivity were detected in Flask H.

When 4.94 x 10^7 dpm of ($^{36}Cl_2$) was used to chlorinate 20g of an unbleached commercial flour (pH 6.2) to pH 4.0, only 0.2% of ^{36}Cl radioactivity was detected in the first trap (Flask G) and 99.8% was associated with the treated flour. Radioactivity in the second trap was negligible.

Fate of available chlorine

Data describing the fate of available chlorine as measured by iodometric titration during flour chlorination are shown

in Table 2. These data indicated that less than 1.5% (1.37, 0.82 and 0.35% for pH levels of 5.25, 4.41, and 4.01, respectively) of the total initial Cl_2 remained as available chlorine. Thus, more than 98% of the $^{36}Cl_2$ used for flour chlorination reacted with organic compounds in the flour or was consumed by oxidation/chlorination reactions. The data obtained from the percentage distribution of available chlorine are, thus, similar to those obtained from the percent distribution of radioactivity (Table 1) at each level of flour pH. For commercial flour (pH 6.2), 39 mg of Cl_2 were required to chlorinate 20g of the sample to pH 4.0; with 99.8% of the Cl_2 reacted with the flour.

Incorporation of ^{36}Cl into flour lipids

Chlorination of lipids and flour lipids occurs (Sollars, 1961; Ghanbari et al., 1983b). In this study, incorporation of (^{36}Cl) radioactivity into chloroform-soluble lipid fraction, when expressed on a per gram of flour equivalent, increased as the pH of flour increased (2.60 x 10^5 dpm at pH 5.25 vs 4.80 x 10^5 dpm at pH 4.01) (Table 3). However, when the data were expressed as the percentage of incorporation with respect to total $^{36}Cl_2$ used, chlorine incorporation did not increase as the pH of flour decreased (33.3% at higher pH vs 27.0% at lower pH). This is believed to have resulted from a decreased rate of total chlorine incorporation into the flour lipids as chlorination progresses. This interpretation is supported by the observation that chlorine incorporation increased in nonpolar lipids and decreased in polar lipids (Table 3) as the pH of flour decreased. Polar lipids generally have a higher degree of unsaturation in their fatty acid residues than nonpolar lipids and according to previously published data, are more prone to chlorination reactions (Ghanbari et al., 1982b; 1983b) to form chlorine addition reaction products and oxidative degradation products.

An increase in the absolute amount of ^{36}Cl incorporation at high chlorination levels was also detected when the unbleached commercial flour was chlorinated to pH 4.0. Chlorine incorporated per lipid equivalent per g flour was 6.18 x 10^5 dpm, while the percent of incorporation with respect to total $^{36}Cl_2$ used was 25%.

The radioactivity associated with chloroform-soluble lipid fractions reported in Table 3 was due to incorporated chlorine, and not chlorine associated by various mechanisms to the various lipid fractions. This is indicated by data showing that a negligible (less than 0.01%) association of $^{36}Cl^-$ with chloroform-soluble lipid fraction in the control runs, where an equal amount of radioactivity used to chlorinate flours was added to untreated flour as $Na^{36}Cl$ during chloroform extraction. Additional evidence supporting chlorine incorporation was the observation that 100% of the radioactivity associated with chloroform-soluble lipids was eluted with organic solvents in column chromatography and TLC, while $^{36}Cl^-$ added to nonradioactive lipid extracts was not eluted with organic solvents.

Table 3—Incorporation of (^{36}Cl) radioactivity into flour lipids after flour chlorination

	(^{36}Cl) incorporation in chloroform-soluble lipid fraction[a]		Percent of ^{36}Cl incorporated into	
Flour pH	dpm X 10^5/g flour[b]	Percent[c]	Nonpolar lipids[d]	Polar lipids[d]
5.25	2.60	33.3	31.2	68.8
4.41	4.40	33.7	32.8	67.2
4.01	4.80	27.0	39.1	60.9

[a] Chloroform-soluble lipids were separated into polar and nonpolar fractions. Experimental conditions as in Table 1.
[b] Radioactivity in chloroform-soluble lipid fraction after chloroform extraction from 1g of flour.
[c] Percent in chloroform-soluble lipids with respect to total (^{36}Cl) radioactivity used for chlorination.
[d] Percent means percent of (^{36}Cl) radioactivity incorporated into each fraction with respect to chloroform-soluble lipid fraction.

Distribution of ^{36}Cl among different fractions of defatted flour

Data describing distribution of ^{36}Cl in the defatted treated flours are shown in Table 4. The predominant form of chlorine in water-soluble fractions was $^{36}Cl^-$: 40.3%, 44.8% and 49.1% in flour samples treated to pH 5.25, 4.41 and 4.01, respectively. The absolute amount of radioactivity incorporated into water-soluble organics appeared to be the same regardless of the level of chlorination. The percentage incorporation relative to the total $^{36}Cl_2$ used decreased with increased chlorination. Water-insoluble fractions, which were predominately protein and starch, contained 17.9 to 21.4% of the radioactivity used to chlorinate the flours.

For the comparative study with commercial flour chlorinated to pH 4.0, the defatted flour was shown to contain 52.2% radioactivity as $^{36}Cl^-$, 2.0% as incorporated into the water-soluble organics and 20.8% incorporated into water-insoluble fraction.

In summary, more than 99% of $^{36}Cl_2$ used to bleach flour reacted with components of the flour. Flour lipids incorporated 27.0–33.7% of the ^{36}Cl depending on the level of chlorination. Chlorine incorporation into the water-soluble organics of defatted, treated flours ranged from 2.1–4.7%, whereas the water-insoluble fraction incorporated 17.9-21.4% of radioactivity. $^{36}Cl^-$, a product of the oxidation reaction of $^{36}Cl_2$, existing mainly in the water-soluble fraction of defatted flour, constituted 40.3–49.1% of the radioactivity. The level of $^{36}Cl^-$ was dependent upon the level of chlorination. While the amount of radioactivity incorporated into different fractions was generally higher at high chlorination levels, the percentage incorporation was approximately equal or lower. Studies are underway to characterize and identify the chlorinated molecules in the lipid fractions of the treated flours. The nature of chlorination reactions of Cl_2 with flour proteins is also being studied.

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Table 4—Distribution of (^{36}Cl) radioactivity among different fractions of defatted flour

	$^{36}Cl^-$ in water-soluble fraction		(^{36}Cl) Radioactivity in			
			Water-soluble organics		Water-insoluble fraction	
Flour pH	dpm X 10^5/g flour[a]	Percent[b]	dpm X 10^4/g flour[a]	Percent[b]	dpm X 10^5/g flour[a]	Percent[b]
5.25	3.14	40.3	3.67	4.7	1.64	21.0
4.41	5.85	44.8	4.04	3.1	2.34	17.9
4.01	8.72	49.1	3.73	2.1	3.80	21.4

[a] Total radioactivity of the fraction derived from 1g of flour.
[b] Percent relative to total (^{36}Cl) radioactivity used for chlorination.

Measurement of Water Activity of Salt Solutions and Foods by Several Electronic Methods as Compared to Direct Vapor Pressure Measurement

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ABSTRACT

The Kaymont-Rotronics Hygroskop DT, the Beckman-Sina and the Protimeter were tested for their ability to measure water activity (a_w) of five foods over the 0.11 - 0.85 a_w range. Each instrument was calibrated at 30 ± 0.1°C against ten saturated salt solutions using a modification of the AOAC Method 32.004-32.009. Regression of the reading versus true a_w, as determined by the vapor pressure manometer (VPM), gave high r^2 values for the saturated salt solutions between 0.32 - 0.85 a_w. An error of approximately 0.01 a_w units was found using the 1 hr data but no significantly better regression line was found using the 24-hr data as compared to 1 hr data. Measurement of the a_w of five foods, however, gave values differing by an average of 0.051 a_w units as compared to the VPM readings. This study demonstrates justification of the FDA cutoff a_w value of 0.85 for low-acid foods as a margin of safety.

INTRODUCTION

THE WATER ACTIVITY (a_w) of many foods is an important thermodynamic property which can be used to predict the state and relative stability of food with respect to physical properties, rates of deteriorative reactions, and microbiological growth (Labuza, 1980). As defined, water activity is the ratio of the partial vapor pressure of water in air in equilibrium with a food to the partial saturation vapor pressure of water vapor in air at the same temperature (Scott, 1957). In recent years the FDA has incorporated the a_w principle in the definition of low-acid foods [21 CFR 113.3(n)] and uses this and other criteria to determine whether a scheduled process must be filed for the thermal destruction of *Clostridium botulinum*. Low-acid foods means any foods . . . with a finished equilibrium pH greater than 4.6 and a water activity (a_w) greater than 0.85.

Methods for measuring a_w have been reviewed by several authors (Prior, 1979; Stoloff et al., 1978; Labuza et al., 1976; Greenspan, 1977). Among the methods tested, the vapor pressure manometric technique (VPM) was found by Labuza et al. (1976) to be the most accurate method for an a_w range of 0.32 - 0.97 as well as the only practical method which gave a direct measure of the water vapor pressure as exerted by the sample. Accuracy for the VPM method is variously reported in the literature. Acott and Labuza reported an accuracy of 0.005 a_w units at values below a_w 0.85. Above 0.85, accuracy fell to 0.02 units due to temperature fluctuations (Labuza et al., 1976). Lewicki et al. (1978) showed improved accuracy by developing an expression to correct for the difference in sample versus gas space temperature. Troller (1983) reported an accuracy of 0.009 a_w units for an a_w range of 0.75 - 0.97 when using the Lewicki correction method for a VPM with a pressure transducer instead of an oil manometer.

Of the many indirect techniques for a_w measurement, the electric hygrometer and the dew point measuring device were utilized in this present study. Electric hygrometers use a sensor based on an electrolyte such as LiCl deposited between two electrodes and measure the change in electrical impedance as a function of absorbed water vapor. These instruments respond rapidly to changes in relative humidity and have the advantage of being portable and easy to use. Reported limitations are the loss of accuracy due to sensor aging, eventual poisoning by absorbed volatiles such as glycols, hysteresis effects at high a_w levels, and need of frequent calibration (Vos and Labuza, 1974). Electric hygrometers have been shown to have good accuracy over a wide range of a_w values. Labuza et al. (1976) showed an accuracy of 0.02 a_w units over the 0.32 to 0.97 a_w range for several electric hygrometers. Troller (1977) reported an accuracy of 0.005 a_w units over the 0.75 - 0.97 a_w range for a Sinascope, with a coefficient of variation not exceeding 1%.

The dew point measuring device tested in this study uses an optical dew point sensory that detects condensate on a mirror while cycling continuously in temperature around the dew point. Any dew formed on the mirror is subsequently reevaporated into the sample chamber which prevents sample moisture changes. The dew point data obtained can then be directly related to the a_w of the sample. Dew point sensing devices are reported to give an accuracy of 0.003 a_w units in the 0.75 - 0.99 a_w range, as reviewed by Prior (1979). At lower a_ws their accuracy falls off because there is not enough vapor in the headspace to cover the mirrored surface and change its reflectivity.

As a prelude to regulatons for low-acid foods which would require accurate measurement of a_w, Stoloff (1978) recommended a procedure for the calibration of a_w-measuring devices as well as recommendations for sample testing. Based on the Stoloff study and the finalization of the FDA regulations mentioned earlier, the AOAC published standardized calibration methods (32.004-32.009) which require use of at least five salts at 25 ± 0.1°C and determining equilibrium by recording measurements at periodic intervals until the difference in a_w is no greater than 0.01 units. In addition, specific instruments suggested for use in determining a_w water activity are listed by manufacturer.

The objectives of this present study were to test three additional devices, not available at the time of the Stoloff study, and to determine their accuracy and reliability for a_w measurement utilizing modifications of the AOAC procedure. In addition, comparisons were made between the VPM and these instruments, pertaining to measurement of saturated salt solutions and food samples, to determine if current Federal regulations are commensurate with instrument capability.

MATERIALS & METHODS

THREE COMMERCIALLY AVAILABLE instruments (two electric hygrometers and a dew point sensing device) were tested for a_w measurement.

The Kaymont-Rotronics Hygroskop DT (Kaymont Instrument Corp., Huntington Station, NY) is an electric hygrometer that operates with a sensory based on a liquid hygroscopic substance. It has a jacketed stainless steel sample holder that holds about 1-5 grams and orients the sensory just above the sample providing a small headspace and accurate temperature control. This instrument also has digital display and two trend indicaors to show when equilibrium has been reached.

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The Beckman-Sina (Beckman Instrument Inc., Cedar Grove, NJ) uses a sensory that has a gel impregnated with an electrolyte. The sensory is operated through a calibrated plug-in range unit within a range of 0.1 - 1.0 a_w. Other ranges can be obtained by substituting appropriate plug-on sensors. The sample container holds from 0.5 - 1.0g and provides a small headspace for a rapid rate of equilibration. Continuous measurement of a_w is traced on a flat-bed recorder so that equilibrium time can be observed.

The Protimeter (Protimeter Ltd., Marlow, England) is a dew point sensing device that utilizes an optical photocell sensor in its operation, as already described. The sensor is enclosed in a chamber that holds about 20 - 40g of sample. The instrument displays both dry bulb and dew point temperature. From a provided chart, the %RH of the headspace can be interpolated.

All three instrument manufacturers suggest that equilibrium occurs in about 60 min and all three instruments give values of percent relative humidity ($a_w \times 100$).

To determine the range capability and calibration sensitivity of each instrument, the a_w of ten saturated salt solutions was measured at 30 ± 0.1°C. All salts used in this study are listed in Table 1, and saturated solutions were prepared according to the AOAC procedure (32.007). Saturation equilibrium of the solutions was checked after storage for 24 hr at 30°C prior to measurement. The salts were stored in sealed jars at 30°C when not in use. The a_w of these solutions was measured on an oil manometer VPM, according to the method of Lewicki et al. (1978). Duplicate measurements were taken at 30°C by each of two technicians. A correction for temperature difference was applied. These data were used instead of the Stoloff (1978) data at 25°C or the Greenspan (1976) data at 30°C since intrinsic errors have been associated with the averaging of literature values in these types of studies, (Labuza et al., 1976). The 30°C temperature was chosen for better temperature control since 25°C would have required refrigeration. The a_w of each salt was then measured on each of the instruments utilizing a random sample order. Duplicate or triplicate measurements were recorded by one to three technicians, after continuous exposure of a sample to a sensor for 1 hr and again after 24 hr. Thus, each salt was measured a maximum of six times on each instrument. The technicians employed in this study had no prior experience with these instruments so that individual biases toward any instrument were eliminated. Data collected from the saturated salt solutions were then fitted to a linear regression of the instrument reading in %RH versus the true a_w, as measured by the VPM in order to calculate the bias and gain where:

$$a_w(100) = (\text{Reading})(\text{Bias}) + (\text{Gain}) \quad (1)$$

Five foods covering a range in a_w from 0.11 - 0.85 were measured in duplicate on each instrument and on the VPM by all three technicians, and the results were compared. However, some samples were measured by only one or two technicians because bias due to sample lot variation was not allowed. The foods utilized in this study, instant dry tea, nonfat dry milk, Parmesan cheese, an intermediate moisture dog food, and grape jelly, were all purchased at a local supermarket. As with the salt solutions, all food samples were preequilibrated to 30°C for 24 hr prior to testing with measurements taken at 1 and 24 hr.

RESULTS & DISCUSSION

TABLE 1 shows the raw a_w values and calculated averages for the saturated salt solutions obtained on the oil manometer VPM at 30°C. The next to the last column shows the Greenspan (1977) values for these same solutions at 30°C. The last column shows data collected by others in our laboratory on a VPM equipped with a pressure transducer sensor, according to the method of Troller (1983), rather than the oil manometer. The results from the oil manometer VPM and those from Greenspan show statistically significant differences at all a_w values when evaluated by the Student t-test at $p = 0.05$. The reason for disagreement in values lies in the methodology used to estimate the true a_ws of these saturated salt solutions. Greenspan's (1977) values were a result of weighted fits to regular polynomial equations as a function of temperature from published data covering a range of 56 yr. Given this range of years and the variation in accuracy of the wide variety of direct and indirect methods of a_w measurement combined in the Greenspan tables, those values can contain significant determinant errors and may provide a poor estimate of the true a_w. These dterminant errors were not introduced in the values obtained on the VPM which directly measures the static physical phenomenon of vapor pressure used to calculate a_w. The standard deviations of the a_ws obtained on the VPM ranged from 0.001 - 0.008 which agree with the results obtained by Labuza et al. (1976). Table 1 shows some differences between the oil manometer VPM values and those values found using the pressure transducer; however, these differences were not found to be significant when subjected to a t-test ($p = 0.05$). While the pressure transducer values might be a more accurate estimate of the true a_w, the oil manometer values were used in the regression calculations to assure uniformity when applying those equations to the food samples which also measured on the oil manometer VPM.

The mean values of the measured raw data, obtained on the instruments for the saturated salt solutions, are summarized in Table 2. The estimates of the true a_w, as measured by the oil manometer VPM, are listed in the first column for comparison. Table 3 shows the accuracy of each instrument as evaluated by the average mean difference of each instrument from the VPM values from the values generated in Table 2. The poor values of accuracy indicate that even in its original condition, some further calibration of each instrument was warranted. This same result was a key point in the Stoloff study, one which manufacturers have left understressed. It is also evident from Tables 2 and 3 that the Protimeter was not at equilibrium at the 1 hr measurement, since a significant change

Table 1—Water activities for saturated salt solutions as measured by the oil manometer VPM at 30°C

Salt	Technician A		Technician B		Average value of two technicians[a]	a_w Value from Greenspan (1977)	Pressure transducer VPM value[b]
	Sample 1	Sample 2	Sample 1	Sample 2			
LiCl	0.116	0.114	0.115	0.116	0.115 ± 0.001	0.1128 ± 0.0024	0.110
CH_3COOK	0.224	0.224	0.224	0.227	0.225 ± 0.001	0.2162 ± 0.0053	0.236
$MgCl_2$	0.327	0.331	0.327	0.331	0.329 ± 0.002	0.3244 ± 0.0014	0.325
K_2CO_3	0.445	0.455	0.444	0.446	0.447 ± 0.005	0.4317 ± 0.0050	0.437
NaBr	0.562	0.578	0.577	0.579	0.574 ± 0.008	0.5603 ± 0.0038	–
$NaNO_2$	0.642	0.657	0.647	0.650	0.649 ± 0.006	NA[c]	0.648
NaCl	0.758	0.774	0.772	0.773	0.769 ± 0.007	0.7509 ± 0.0011	0.748
KCl	0.847	0.851	0.849	0.853	0.850 ± 0.003	0.8362 ± 0.0025	0.841
$BaCl_2$	0.917	0.931	0.914	0.917	0.920 ± 0.008	NA[c]	–
K_2SO_4	0.965	0.989	0.976	0.980	0.977 ± 0.001	0.9700 ± 0.0040	–

[a] ± standard deviation
[b] Standard deviations were ±0.001 for all salts
[c] NA — not available

Table 2—Measured mean values of a_w for saturated salt solutions at 30°C

Salt	VPM[a]	Kaymont-Rotronics[b] 1 hr	Beckman-Sina[b] 1 hr	Protimeter[b]	
				1 hr	24 hr
LiCl	0.115 ± 0.001	0.167 ± 0.001	0.115 ± 0.001	0.175 ± 0.001	0.175 ± 0.001
CH_3COOK	0.225 ± 0.001	0.260 ± 0.002	–	–	–
$MgCl_2$	0.329 ± 0.002	0.367 ± 0.003	0.275 ± 0.005	0.344 ± 0.001	0.344 ± 0.001
K_2CO_3	0.447 ± 0.005	0.492 ± 0.003	0.460 ± 0.002	0.486 ± 0.010	0.456 ± 0.007
NaBr	0.574 ± 0.008	0.614 ± 0.001	0.568 ± 0.002	0.691 ± 0.010	0.604 ± 0.008
$NaNO_2$	0.649 ± 0.006	0.668 ± 0.003	0.637 ± 0.003	0.634 ± 0.010	0.642 ± 0.006
NaCl	0.769 ± 0.007	0.768 ± 0.004	0.750 ± 0.001	0.738 ± 0.007	0.764 ± 0.006
KCl	0.850 ± 0.003	0.839 ± 0.005	0.832 ± 0.001	0.830 ± 0.008	0.865 ± 0.006
$BaCl_2$	0.920 ± 0.008	0.896 ± 0.002	0.860 ± 0.002	0.865 ± 0.009	0.917 ± 0.006
K_2SO_4	0.977 ± 0.001	0.937 ± 0.001	1.00 ± 0.001	0.944 ± 0.008	1.00 ± 0.005

[a] Measured mean of 4 replicates.
[b] Measured mean of 6 replicates.

in the mean value had occurred in 24 hr. No significant decreases (t-test, p = 0.05) were found for the Kaymont-Rotronics or Beckman Instruments during the 24 hr period; thus only 1 hr measurements are listed.

Listed below are the linear regression equations for calibration of the three instruments using the 1 hr readings over the whole a_w range tested.

Kaymont-Rotronics:
$a_w(100) = 1.106$ (reading) $- 7.879$ $\quad r^2 = 0.996$ (2)

Beckman-Sina:
$a_w(100) = 0.991$ (reading) $+ 1.577$ $\quad r^2 = 0.988$ (3)

Protimeter:
$a_w(100) = 1.121$ (reading) $- 9.342$ $\quad r^2 = 0.966$ (4)

As seen from the r^2 values, all three instruments produced equations showing high linear correlations. It should be noted that the slopes were close to one but the intercepts were not zero, which was the main cause of error. In developing the calibration curves, it was found that above a_ws of 0.85 and below 0.32 the instruments were less linear, perhaps indicating physical factors in the sensor which are dominating. This can be seen by the decrease in the average mean difference from the VPM values when evaluating between 0.32 and 0.85 a_w, as shown in Table 3. This justifies using a minimum of five points for calibration as recommended by Stoloff (1978), especially in an a_w range close to that of the test food. This would correct for sensor limitations at the extreme a_w ranges. It must also be stressed that these equations pertain only to each individual instrument; any device of the same model will have its own particular equation. No significantly better regression lines (t-test, p = 0.05) were found using the 24 hr data rather than the 1 hr data for the Beckman instrument or the Kaymont. While the Protimeter showed a significant difference below 0.32, this can be attributed to the slow response of the dew point sensor in the low relative humidity regions (<0.32 a_w). These results, however, suggest that even though these three instruments may not come to complete equilibrium at 1 hr, one would not need

Table 3—Comparison of overall mean difference in a_w between electronic a_w devices and the VPM method

a_w Range	Kaymont-Rotronics 1 hr	Beckman-Sina 1 hr	Protimeter	
			1 hr	24 hr
0.11 – 0.98	0.031	0.022	0.043	0.019
0.32 – 0.85	0.026	0.020	0.040	0.014

to run multiple calibration curves for different times of exposure if the proper a_w calibration range is used. The a_w value generated from the 1 hr curves for an instrument reading of 85% RH would thus be 0.843 for the Kaymont, 0.858 for the Beckman, and 0.836 for the Protimeter. The difference for the two electric hygrometers is within the ±0.01 value established by the regulatory guidelines. Unfortunately, the difference for the dew point device is 0.014 a_w units, which exceeds the 0.01 value. This is a result of the large negative gain found, which can be dominated by the errors for the low a_w values mentioned before.

The a_w results for the food samples listed in Table 4 showed large deviations in comparison to the VPM. Interestingly, the average standard deviation for all samples was ±0.003 a_w units when measured by the instruments, as compared to ±0.007 when measured by the VPM. However, the instruments gave very different values. The difference between the VPM value and the calculated a_w from the instrument reading varied with the samples by as much as ±0.14 a_w units to as low as ±0.001, but the variation was different for each instrument and food combination. On the average, the difference was ±0.051 a_w units for the instruments. These errors suggest that the FDA criterion of using 0.85 for the low-acid food cutoff may be reasonable even if *Clostridium botulinum* does not produce toxin below an a_w of 0.92, since the food samples in this study showed an inexplicably large deviation in measured a_w values as compared to those found by the VPM. This brings into question what is actually being measured by

Table 4—Corrected measured a_w values of foods at 30°C

Food	VPM	Kaymont-Rotronics		Beckman-Sina		Protimeter	
		Measured	Δ	Measured	Δ	Measured	Δ
Instant Tea	0.130 ± 0.007	0.174 ± 0.002	+0.044	0.192 ± 0.002	+0.062	0.267 ± 0.002	+0.137
Nonfat Dry Milk	0.137 ± 0.004	0.203 ± 0.002	+0.066	0.272 ± 0.003	+0.135	0.277 ± 0.004	+0.140
Parmesan Cheese	0.693 ± 0.007	0.725 ± 0.006	+0.032	0.713 ± 0.003	+0.020	0.721 ± 0.003	+0.028
Grape Jelly	0.802 ± 0.009	0.852 ± 0.002	+0.050	0.818 ± 0.004	+0.016	0.820 ± 0.003	+0.018
IMF Dog Food	0.814 ± 0.009	0.800 ± 0.003	–0.014	0.749 ± 0.003	–0.065	0.815 ± 0.005	+0.001

these instruments for a complex food system. However, it must be remembered that the results presented were based on a comparison with the VPM and that no other direct method of water activity measurement exists.

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lecithin had not shown mold damage after the 10-day period except in the case of sucrose-chocolate samples. Even in the latter case, the spread of mold growth in the sample containing the lower concentration of surfactant was considerably greater than in the 1.0% lecithin sample. A striking observation was the absence of mold damage in both L-sorbose samples.

Although L-sorbose is not fermented by oral bacteria (Bussière, 1976), it is not clear whether this resistance is only to fermentation by bacteria or indeed other microorganisms. However, L-sorbose would appear to have very good humectant properties, comparable to sucrose, from the foregoing results. This is in agreement with previous claims (Bussière, 1976) that tests at 66% and 80% RH showed that sucrose, L-sorbose and mannitol exhibited the least hygroscopicity while β-D-fructose and sorbitol were the two most hygroscopic of several carbohydrate sweeteners compared.

L-sorbose has been found also to compare very well with sucrose in chocolate sensorically (Ogunmoyela and Birch, 1983). It may, therefore, be used as a replacement for sucrose. Its use in food products has hitherto been limited by earlier claims that it is undesirable as a food sugar because of its poor clinical tolerance and tendency to cause physiological modifications such as plasmolysis. However, work by Dupas (1974) has shown conclusively that L-sorbose is of excellent clinical tolerance and causes no biologically adverse effects. It has been further shown to be utilizable especially in sweet food products, like candies, for the purpose of preventing dental caries (Bussière, 1976), while L-sorbose-sweetened chocolate conched very well and presented the same characteristics as traditional chocolate from sucrose, producing even a slightly lower viscosity than sucrose-chocolate, which facilitates the molding operation. In contrast, the use of β-D-fructose and sorbitol particularly in sweetener mixtures for chocolate or other food products must be carried out restrictively and only with adequate knowledge of the storage properties of such mixtures.

It is possible therefore that perhaps in time, the sensory and physicochemical advantages of L-sorbose in chocolate will make it a significant competitor for sucrose.

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Zn & Fe BIOAVAILABILITY FROM WINGED BEAN FLOUR . . . From page 1135

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A New Mapping Super-Simplex Optimization for Food Product and Process Development

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ABSTRACT

Graphic illustration of response surface is difficult to carry out for cases with more than two factors as the graphs are of higher orders than three dimensions. An approximation was made by dividing the level values of each factor into four groups of equal intervals, and then the response values plotted were linked to each other when all other factor levels were "in the same groups" of experimental conditions, instead of "under the same conditions" in the case of two or three dimensional graphics. When applied to mathematical models, this new mapping simplex optimization significantly ($p < 0.01$) reduced the number of experiments required for reaching the optimum as compared to the Morgan-Deming simplex optimization. Most experiments on food analysis and processing have been optimized within 25 - 35 iterative experiments depending on the number of factors. Computer-directed optimization in conjunction with the mapping technique followed by a simultaneous shift of factor levels greatly facilitated research supervision.

INTRODUCTION

SINCE MAN cannot usually predict the possible effects of changing more than two factors simultaneously during experiments, especially when the factors are interacting, optimization techniques, e.g., simplex optimization, are useful in improving research efficiency.

Simplex optimization is an automated, stepwise process for experimental designs. Once the range for each experimental condition (factor) is initially set, the subsequent experimental conditions are generated by a computer, thus all experiments can be automatically guided toward the optimum with minimum effort. The basic principle of simplex optimization is to move away from the experiment (vertex) which has yielded the worst result (response) in each simplex consisting of (n + 1) experiments, where n is the number of factors. The procedure to find the next experimental conditions which move the experiment in the opposite direction from the worst in the previous simplex can be programmed in a hand-held computer or a programmable calculator. Our experience has shown a great improvement in research efficiency, as lab technicians or students can carry out entire experiments efficiently and with minimum supervision once the factor ranges have been decided initially through discussion with their supervisor.

In the previous paper (Nakai, 1982), a modification was proposed for the super-simplex optimization (SSO) of Routh et al. (1977) to avoid a problem of the search stalling at the boundary set as a constraint. This modified super-simplex optimization (MSS) was more efficient in reaching optima than other simplex optimization techniques. However, no improved efficiency was obtained when the quadratic regression equation did not fit the experimental response surface.

It is a general trend in iterative optimization procedures that the speed of approaching the optimum is quicker at the beginning and progressively slows thereafter. To obtain further improvement in efficiency a mapping procedure was incorporated into the SSO to expedite the later stage of the optimization after a certain number of iterative experiments according to the SSO algorithm.

Techniques for graphic illustration of data of higher orders than three dimensions are unknown. This means that response surfaces composed of more than two factors are impossible to visualize graphically. The approximate drawing of the response surface of multidimensional data was attempted in this simplex optimization. When applied to mathematical models, this new mapping super-simplex optimization (MSO) significantly improved the optimization efficiency as compared to the simplex optimization (MDS) of Morgan and Deming (1974), even for those mathematical models for which MSS was not efficient previously (Nakai, 1982).

This paper describes details of the MSO procedure and the results of comparing the optimization efficiency with the MDS method. Examples of the application of this new optimization technique to food product and process development are also demonstrated.

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MATERIALS & METHODS

Procedural outline

An outline of the optimization procedure is schematically illustrated in Fig. 1.

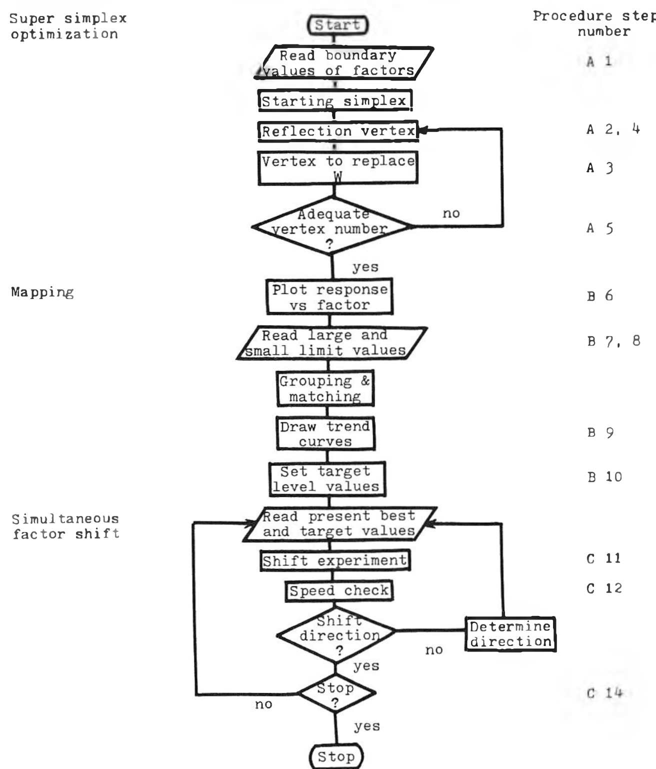


Fig. 1—Outline of the mapping super-simplex optimization.

Mapping principle and its computer program

As graphic illustration of multidimensional data, i.e. higher orders than three dimensions, was difficult, an approximate visualization of the experimental response surface was attempted for demonstrating the trend toward the optimum.

For each factor, the level values used in optimization are divided into four groups based on their locations on a scale within large, medium and small limits (grouping). Division into more than four groups was tried to improve the accuracy of locating the optimum; however, this was abandoned since the available number of data for mapping decreased.

The large and small limits are determined from a plot of the response value against each factor level so as to include all data points and only major points, respectively. The ranges of the two limits can be quite different when the data points are localized in the scattergraph. The medium limit is calculated by the computer to be the average of the entered values of the large and small limits. These three different limits are used for grouping to increase the frequency of data linking so that adequate information on the location of the optimum is obtained.

From the grouped data, matched data to be linked are found. Data points entitled to be joined for one factor are those that belong to the same groups of the other factors. A hypothetical three factor case is illustrated in Fig. 2. When the response values are plotted against factor 1 (Fig. 2a), the possible location of the optimum is not easy to conjecture. In Fig. 2c and 2d, the data points are grouped. For factor 2, data points 5, 7, 10, 12 and 13 belong to the same group (third from left in Fig. 2c), and for factor 3, data points 7, 10, 11 and 13 belong to the same group (second from left in Fig. 2d). Since data 7, 10 and 13 are common in these two groups of data, implying that they have been obtained under approximately the same conditions with regard to factors 2 and 3, these points are entitled to be linked to estimate the response surface in the response vs factor 1 plot (Fig. 2b). Other data points in the above grouped data, i.e. data 5, 11 and 12, are not entitled to join this linked group since they have been obtained through experiments carried out under different "condition groups." Data points 5 and 12 can be linked separately as they belong to another group for factor 3 (fourth from left in Fig. 2d). After the mapping procedure has been completed by linking the other points (6, 8 and 9; and 1 and 2 in Fig. 2b), the approximation of the optimum is clearer (Fig. 2b) than before (Fig. 2a). It is highly probable that the level of factor 1, for the optimum locates between the level values for vertices 6 and 13.

From the maps for all factors the target level values are estimated. The target value of a factor in conjunction with the present best value of the factor will show the most probable area where the optimum may be located. Although the target setting is absolutely dependent on an individual's judgement, an example of the target setting rule which has brought about successful optimization in the past is illustrated in Fig. 3.

A computer program for combined grouping and matching was written for the UBC Amdahl 470 V/8 computer and a Sharp PC 1500 hand-held computer.

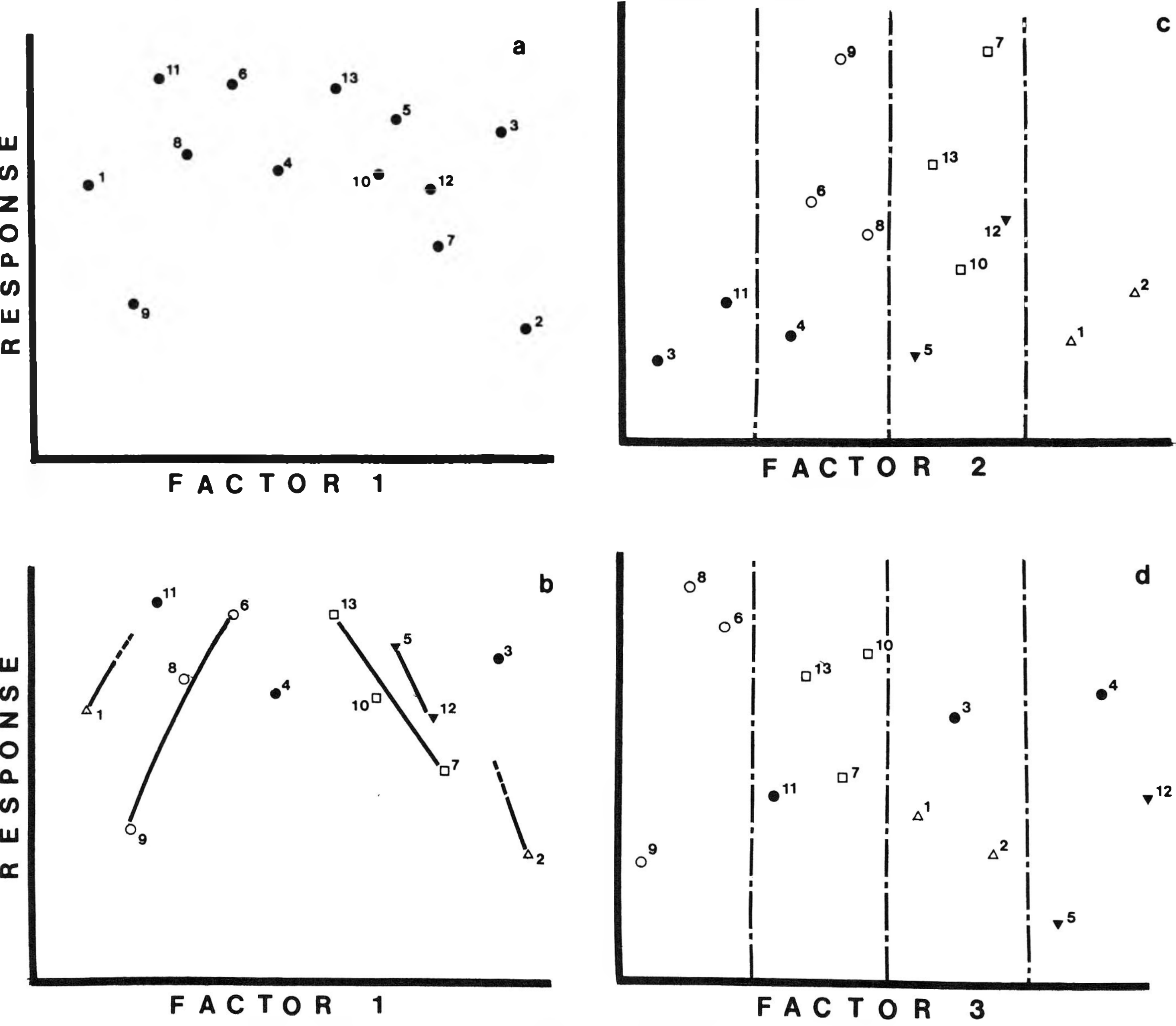


Fig. 2—Hypothetical graphs for explaining the mapping principle (maximization).

Simultaneous factor shift program

The computer programs written for a Monroe 1880 programmable calculator and Sharp PC 1500 computer are designed to shift the factor level 1/5 the distance between the present best (B) and the target values (T) starting from the B value. The shift is carried out for all factors simultaneously and the program provides three extra shift values beyond the T values to prepare for cases when the T values are erroneously set too close to the B value. An example of the simultaneous factor shift is as follows: if the B and T values for pH are 6.0 and 7.0, and for temperature 20 and 30°C, the shift experiments are designed as exp. 1 (pH 6.2, 22°C), exp. 2 (pH 6.4, 24°C) . . . exp. 5 (pH 7.0, 30°C) . . . exp. 8 (pH 7.6, 36°C).

Optimization of mathematical models

Mathematical models used in the previous paper (Nakai, 1982) were again used for objective comparison of optimization algorithms after slight modification (Table 1). For the models for which the theoretical factor values were 0 at the optimum, variable x was substituted by (x + a) to avoid accidental optimization due to erroneous operation of the computer programs.

The SSO program was also modified from $c > 2$ (Fig. 2, Nakai, 1982) to $c > 0.65$ for the extension conditions to increase the flexibility of the search thus avoiding the stalemate of the search at the boundaries. The following procedure was finally adopted.

A. Super simplex optimization

1. Read the boundary values of each factor for the initial simplex program to obtain (n + 1) vertices where n is the number of factors.
2. Enter the response values of (n + 1) experiments into the program to calculate the reflection vertex.
3. Enter the response value into the program for computing the vertex to replace the worst value (W) within the simplex.
4. Form a new simplex by replacing W with a new vertex (better one of two vertices obtained in steps 2 and 3) and continue optimization by repeating steps 2, 3 and 4.
5. After carrying out 18 - 20 experiments, 20 - 22 experiments and 22 - 24 experiments for 3, 4 and 5 factor cases, respectively, transfer to the next mapping procedure.

B. Mapping

6. Plot the response values obtained against the factor levels on separate graphs for each factor.
7. Determine the large and small limits of factor levels for each factor. The large limit is taken as the lower and upper factor levels that include all data points while the small limit includes only the majority of data points.
8. Enter these limit values into the grouping-matching program to obtain a series of matched data.

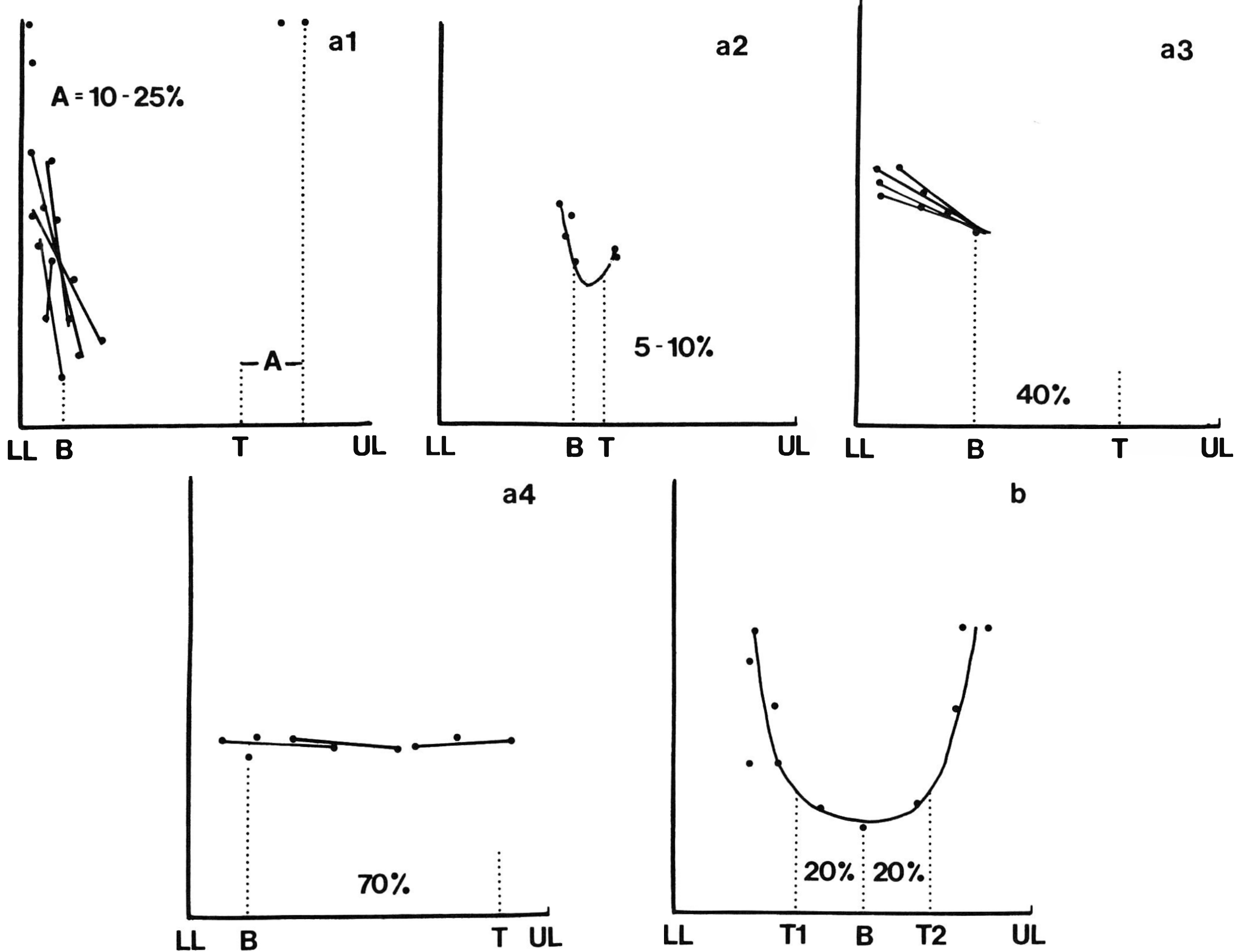


Fig. 3—Examples of target level setting (minimization): (a1) Stalled optimization. Target value is set 10 - 25% of the range (the distance between LL and UL) from the point furthest from the stalled points. B, current best value; T, target value; LL, UL, original lower and upper limits. (a2) Slow movement towards the optimum in a sharp concave. Target value is set 5 - 10% of the range from the current best value. (a3) Medium movement towards the optimum linearly. Target value is set 40% of the range from the current best value. (a4) Fast movement towards the optimum with a straight curve. Target value is set 70% of the range from the current best value. (b) Direction of movement towards optimum is unclear. Target values are set ±20% of the range on both sides of the current best value.

9. Join the matched data points on each graph or draw most probable lines which indicate where the optimum is most likely to be located.

10a. Set target level values for each factor such that the optimum is most likely to occur between the best value found in "A" above and the target value (Fig. 3).

10b. Set targets on both sides of the present best value for graphs where it is difficult to determine which direction the search should be continued (Fig. 3-b).

C. Simultaneous factor shift

11. Shift experiment. Continue the experiments according to the shift program until the response value becomes worse than the preceding one. For factors with two targets on both sides of the present best value (10b) no shift experiment is performed until step 13.

12. Speed check for fast shifting factors (where the target value is set far from the present best value) or for factors whose target values are unclear. Using the best vertex from step 11, do experiment by changing only the factor in question to the level value calculated for the next vertex in the shift program. If the response vlaue is worse or better than the preceding best response value, then the target value should be changed to decrease or increase the distance from the present best level.

13. Direction determination (case 10b). Do experiments by changing the factor level to the target values on either side of the best value. Decide the direction of search depending on which side gives the better response value. When two factors belong to case 10b, try all combinations of target values since there may be interactions between factors.

14. Termination. Depends on the objective of the optimization but in general, the search is terminated with the response value has converged on the extremum with only slight fluctuations, e.g., the same response value is repeated three times.

Comments

(a) If the initial boundaries are very broad, a larger number of experiments may have to be carried out during super-simplex optimization. Super-simplex optimization should be continued until the responses from the last three vertices to replace the worst immediately before the mapping are within 10% difference from each other.

(b) Obtaining good graphs which give a clear indication of the shift direction toward the optimum is extremely important. If good graphs are not obtained the following may be tried: (i) Continue super-simplex optimization for several more vertices and incorporate these data into the mapping procedure. (ii) Change the small and large limit values for grouping.

(c) If, in the shift experiment, the first experiment gives a response value worse than the present best response, change the direction of the factor which is most uncertain of its shift direction. The shift experiment is repeated with a new shift program.

(d) For factors with almost no response improvement, set the level values of these factors on the side opposite to where the present best value is located, then continue the shift experiment. Later, direction determination of these factors may be done.

Table 1—Comparison of optimization efficiency

	Vertex number of optimization	
	MDS	MSO
Model (1) $y=20-5x_1-2x_2-4x_3-6x_1x_2-x_1x_3+3x_2x_3+4x_1^2+4x_2^2+x_3^2$ (minimization)		
	63	36
	58	19
Model (2) $y=1.34+33.6x_1+25x_2+34.4x_3+22x_4+7x_5-4x_1x_2-12x_1x_3-10x_1x_4-4x_1x_5-16x_2x_3-12x_2x_4-6x_2x_5-15x_1^2-18x_2^2-20x_3^2-26x_4^2-10x_5^2$ (maximization)		
	66	32
	74	55
	98	88
Model (3) $y=\{100[x_3-10\theta(x_1,x_2)]^2+[\sqrt{x_1^2+x_2^2}-1]^2+x_3^2\} \times 2+10.00$ where $2\pi\theta(x_1,x_2) = \arctan(x_2/x_1)$ when $x_1>0$, $= \pi+\arctan(x_2/x_1)$ when $x_1<0$ (minimization)		
	145	48
	85	43
	57	49
	180	27
Model (4) $y=\{(x_1+10x_2)^2+5(x_3-x_4)^2+(x_2-2x_3)^4+10(x_1-x_4)^4\} \times 2+10.00$ (minimization)		
	77	45
	97	33
	73	54
	42	25
Model (5) $y=(x_1^2+x_2^2+x_3^2+x_4^2+x_5^2) \times 100+10.00$ (minimization)		
	84	39
	101	40
	67	32

RESULTS

An example of the simultaneous shift procedure

To visualize the complicated manipulation of the simultaneous shift procedure, the optimization result of model 5 in Table 1 is shown in Table 2. First, the simultaneous shift was carried out using the best level and target level values obtained from the mapped graph for each factor. (The data from 24 vertices performed according to the super-simplex optimization were used for mapping.) After three shifts the response value started to increase, thus the shift was discontinued. Then, the speed check was carried out by changing each factor from the current best (vertex 26) one at a time to the next value calculated by the shift program. These results indicated that the speed of shift of X_2, X_3 and X_5 should be decreased and X_4 should be increased as the response values resulting from these changes are worse and better than vertex 26, respectively.

The direction of the shift was checked based on the best response from the speed check (vertex 31) for all factors, except for X_1 for which the direction of shift was self-evident in the mapped graph. Because of the improved response values obtained from X_3 and X_5 (vertices 34 and 36), their possible interaction was investigated (vertex 37). Using the results of the speed check and direction determination, new target level values were set. For X_3 and X_5, the direction of shift was changed since their response values were better than that found in the speed check. The second simultaneous shift reached the minimum value of 10.03 after 39 vertices for which the MDS required 84 vertices to reach the same minimum value.

Comparison of optimization efficiency

As reported previously (Nakai, 1982), mathematical optimization models were used for objective comparison. These models were also used in this paper. Since different optimization techniques were compared in the previous paper, the mapping super-simplex optimization results were compared with only those of MDS. Special interest was focused on comparison of MDS and MSO using the mathematical models for which the MSS suggested previously (in which a quadratic fitting was incorporated into super-simplex optimization) failed to improve the optimization efficiency over MDS (models 3 and 4 in Table 1), due to inadequate fitting of the quadratic equation used in the MSS to the response surfaces of the models.

As seen in Table 1, when MDS needs a large number of vertices the MSO remarkably improves the optimization efficiency. For fair comparison, the MSO search was terminated when the same optimum response value as that obtained by MDS was reached. A paired sample t-test showed a highly significant ($P < 0.01$) difference in the optimization efficiency between the two methods.

It was found that SSO was more suitable than MDS for initial simplex optimization since SSO yielded data with greater variability. This resulted in a quicker approach to the real optimum in mapping although, compared to MDS, the speed of approach was more quickly reduced at the later stage of optimization.

Examples of application to food processing projects

Two examples are shown. Example 1 required the application of the simultaneous factor shift procedure after the mapping. Most other examples of analytical and processing experiments carried out in our laboratory reached the optima even before entering the mapping stage, therefore the simultaneous shift was not required. Example 2 is different in that it shows two local optima.

Example 1. Viscosity of casein solution.

Casein forms a gel in the presence of calcium, orthophosphates and pyrophosphates when heated. This gel can be utilized for preparation of gelled dessert foods. Prior to simplex optimization, nine experiments were carried out using fractional factorial designs for the above three additives as factors with three levels. The purpose of these experiments was to define the ranges of these additives for the subsequent optimization. Viscosity of the heated casein solution was measured by a Brookfield viscometer. After 14 experiments using SSO, the experimenter erroneously stopped the iteration due to a misunderstanding of step A 5. Mapping from these 14 data did not yield useful graphs due to lack of the number of data. This is because the last three vertices to replace the worst (vertices 10, 12 and 14) have a 23% difference in the response size. This size of difference is larger than the recommended 10% [comment (a)].

Mapping was, therefore, carried out after combining nine data from factorial analysis with the 14 data from SSO as seen in Fig. 4. From these results, a target value for each factor was set as follows (the present best value is shown in parenthesis): $CaCl_2$ 17.9 (15.4) mM, orthophosphates 6.6 (9.6) mM and pyrophosphates 15.8 (12.7) mM. After six subsequent experiments using simultaneous shift, a viscosity of 520 mPa.s was obtained by heating 10% sodium caseinate at 60°C for 20 min in the presence of 16.4 mM $CaCl_2$, 8.4 mM K_2HPO_4-KH_2PO_4 equimolar mixture and 14.0 mM $Na_4P_2O_7$-$Na_2H_2P_2O_7$ equimolar mixture.

Example 2. Milk protein coprecipitation

Coprecipitation of protein from skim milk was maximized using SSO, MSO and response surface analysis (RSA). The factors varied were pH 2 - 7, solids content 2 - 36% and $CaCl_2 \cdot 2H_2O$ 0 - 500 mg/100 mL. All samples were heated for 10 min in boiling water. The SSO terminated by itself after seven experiments at pH 3.17, 29.1% milk solids and 117.9 mg $CaCl_2 \cdot 2H_2O$. The MSO, after 32 experiments, showed the presence of two close maxima at pH 3.17, 29.1% milk solids, and 117.9 mg $CaCl_2 \cdot 2H_2O$ and at pH 6.70, 34.1% milk solids and 471 mg $CaCl_2 \cdot 2H_2O$ with over 90% precipitation. Whereas, the RSA, with 17 experiments, calculated the location of optimum to be at pH 3.71, 2.3% milk solids with 850 mg $CaCl_2 \cdot 2H_2O$ with lower than 60% precipitation.

These results revealed the usefulness of MSO since it was feasible to demonstrate the presence of two local optima. On the contrary, SSO lacked search capability while a maximum found by RSA was inferior to that found by MSO probably because of difficulty in fitting the quadratic equation to a case with two local optima. The mapping of all data obtained during the entire MSO procedure gave a clear vision of the true response surface which was useful for designing practical processing conditions, e.g., for avoiding high acidity in this coprecipitation experiment by selecting pH 6.70 instead of pH 3.71.

DISCUSSION

USEFULNESS of simplex optimization in food research was demonstrated in a previous paper (Nakai, 1982). The new mapping super-simplex optimization has been applied to more than 20 food processing experiments. Frequently, the optima were reached even before mapping. The mapping procedure in these cases was still quite useful since the overview of the response surface around the optimum assisted the subsequent processing operation.

For the final search after the mapping process, it is possible to continue another iterative search using SSO or MDS instead of the simultaneous factor shift; the latter was more efficient in optimization. A drawback of this simultaneous shift plan is the difficulty in writing a complete computer program (steps C 11-14 in Fig. 1). At present the speed check (step C 12) and the direction determination (step c 13) are performed manually.

Table 2—An example of the simultaneous shift procedure (minimization)

	Vertex	X_1	X_2	X_3	X_4	X_5	Response
	Range	1.77	−0.37	0.19	1.02	−0.51	(present best)
Simul. shift (I)		1.61	−0.2	0.3	1.1	−0.42	(target)
Shift 1	25	1.74	−0.34	0.21	1.04	−0.49	10.56
2	26	1.71	−0.30	0.23	1.04	−0.47	10.41
3	27	1.67	−0.27	0.26	1.07	−0.46	10.71
Speed check							
X_1	28	1.67	−0.30	0.23	1.05	−0.47	10.41
X_2	29	1.71	−0.27	0.23	1.05	−0.47	10.52
X_3	30	1.71	−0.30	0.26	1.05	−0.47	10.61
X_4	31	1.71	−0.30	0.23	1.07	−0.47	10.29
X_5	32	1.71	−0.30	0.23	1.05	−0.46	10.54
Direction determination							
X_2	33	1.71	−0.34	0.23	1.07	−0.47	10.41
X_3	34	1.71	−0.30	0.21	1.07	−0.47	10.18
X_4	35	1.71	−0.30	0.23	1.04	−0.47	10.41
X_5	36	1.71	−0.30	0.23	1.07	−0.49	10.22
X_3,X_5	37	1.71	−0.30	0.21	1.07	−0.49	10.12
	Range	1.71	−0.30	0.21	1.07	−0.49	
Simul. shift (II)		1.65	−0.25	0.19	1.15	−0.52	
Shift 1	38	1.70	−0.29	0.21	1.09	−0.50	10.03
2	39	1.69	−0.28	0.20	1.10	−0.50	10.04

For complete automated optimization where the optimization efficiency and the boundary constraint are not strictly required, e.g., molecular weight distribution computation (van de Voort et al. 1979), the MDS algorithm can be recommended. By allowing the search to freely violate the boundary constraint during several early vertices, where feasible, the stalling problem can be circumvented.

Because of the exponential increase of vertex number for optimization as the number of factors increased, the entire MSO program was written to handle up to five factors. Preliminary factorial design experiments are thus important for selection of factors which are really critical for optimization as already described previously (Nakai, 1982).

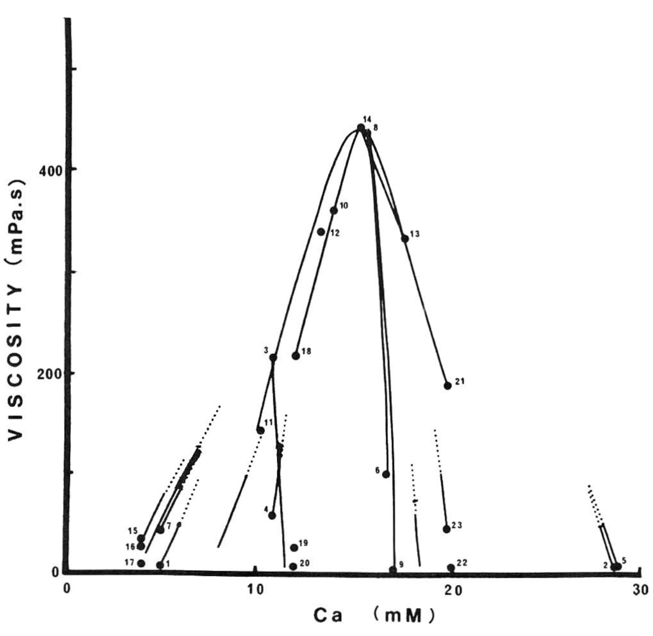
The MSO programs have also been written for a handheld computer (Sharp PC-1500) for ready application of the optimization technique at remote places far from costly main frame computers. The programs, written in Basic language, are available on request.

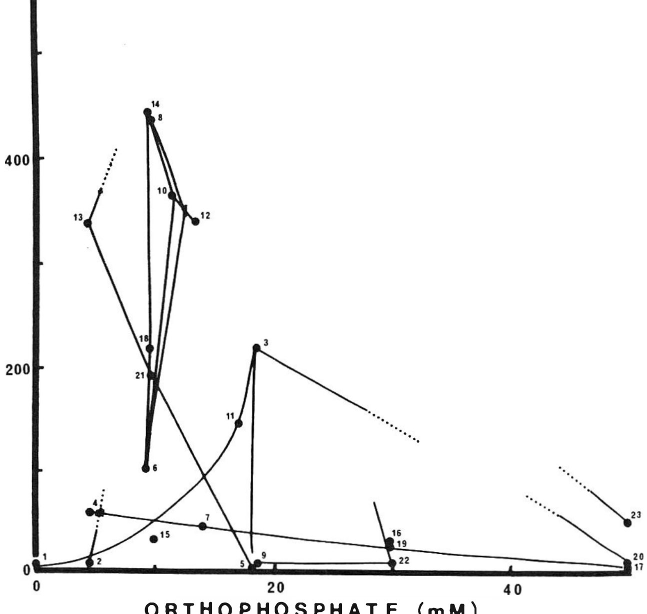
When the precision of response measurement is unsatisfactory, the standard deviation of the response values of the final simplex should be used for terminating the search as Morgan and Deming (1974) suggested.

When more than two responses are to be optimized, arbitrary weights are usually placed on the responses depending on their importance, then the total response values are optimized (Morgan and Deming, 1974). We found that repeating the optimization using each response, one at a time, is more useful instead of introducing inaccurate, subjective weight values. In practice, the first optimization process is carried out using the first response, then the second optimization process to optimize the second response is initiated using a narrower level range of each factor selected from the mapped graph so as to restrain the response values within the desirable range.

In conclusion, a new mapping simplex optimization technique was introduced for improving the optimization efficiency. Mapping and simultaneous factor adjusting procedures were incorporated into the super-simplex optimization algorithm. This mapping procedure, which provided a perspective capability to the iterative search of simplex optimization, proved to be useful in experimenting for food research and development. The computer-assisted administration of research projects as explained in this study has markedly improved the efficiency of conducting research.

—Continued on page 1170





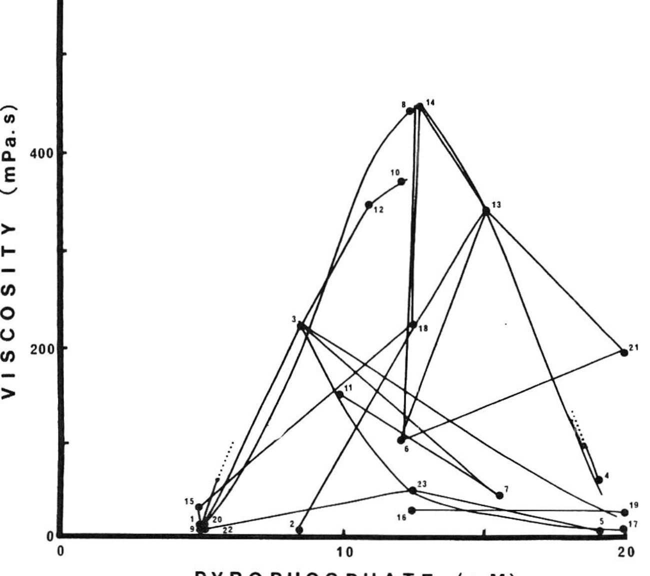


Fig. 4—Mapping results of experiments to maximize viscosity of casein solutions: 10% sodium caseinate solutions were heated at 60°C for 20 min in the presence of $CaCl_2$, K_2HPO_4-KH_2PO_4 equimolar mixture and $Na_4P_2O_7$-$Na_2H_2P_2O_7$ equimolar mixture. Mapping was carried out using 14 data from super-simplex optimization (No. 1-14) and 9 data from factorial design experiments (No. 15-23). The ranges initially set for optimization: $CaCl_2$ 5 - 30 mM, orthophosphates 0 - 50 mM, and pyrophosphate 5 - 20 mM.

Influence of Freezing and Thawing on the Egg Vitelline Membrane

PIERRE ROGER VIAUD

ABSTRACT

The original method finalized for this study was a direct comparison of the isothermic dehydration rate between two membrane samples, one subjected to a freezing-thawing cycle, the other being untreated control. Freezing entailed irreversible modifications in the properties of the vitelline membrane of the hen's egg as indicated by a significant increase in the water absorption capacity; an accelerated drying rate; and a marked decrease in the water retention capacity. Through this method, the cryoprotective action of glycerol at the membrane level was also demonstrated.

INTRODUCTION

TRANSPORT OF WATER into and through cellular membranes and consequent alterations in these membranes are important in the freezing and thawing of biological material. It is difficult to quantitatively measure these effects in tissue or suspended cells because the cells involved are very small. The vitelline membrane array which separates the yolk and the white in the hen's egg, on the other hand, has a suitable albeit complex, multilayer membrane structure which is (a) large enough to permit measurement of these effects through the use of control and variably treated membrane samples obtained from the same egg; (b) strong enough to be separated from the adjacent yolk and white and mounted in suitable sample holders; and (c) readily available.

The array, according to the electron microscope observations of Bellaire and Harkness (1963), consists of: an inner layer about 2.7 μm thick; an outer layer which, because it contains a variable number of sublayers, has a thickness varying between 3 and 8.5 μm; and a continuous membrane which lies between these layers, is about 0.05 - 0.1 μm thick, and contains the phospholipid bilayer making up the membrane proper.

The dehydration rate is directly related to kinetic factors depending on the membrane structure and properties, and to the quantity of free water in the sample or to the activity of this water. Therefore, if a freezing-thawing cycle modifies the drying rate of biological membrane, the activity of the water has varied and/or deep changes have been induced in the structures of the membrane as suggested by Bresson (1978) and Viaud (1978).

The purpose of this study is to show the effect of freezing on the properties of the egg vitelline membrane through a comparison of isothermic dehydration rates between two wet samples of the same origin, one having gone through a freezing-thawing cycle, the other being the control.

MATERIALS & METHODS

Freezing material

The vitelline membrane used constitutes a model which is similar to the cellular structure of a biological membrane separating two continuous quasi-identical environments such as an intra- and extra-cellular media. The water environment selected is the usual physiological serum (0.9% NaCl). The first set of experiments was conducted with pure water; in the second set, physiological serum was used to obtain a more realistic biological model. There were no noticeable differences in the observed results.

The membrane was subjected to a freezing-thawing cycle similar to those used for frozen food commodities. Observations were made of the change with time of the residual water content and water loss rates during drying as compared to a control sample which was not frozen. This technique also made it possible for the cryoprotective action of glycerol to be checked.

Freezing cells

Sample-holders were composed of two perspex rings (1 cm in radius) which, when placed together encircled the membrane sample. This system provided for satisfactory stretching of the membrane which, with a minimum of care, can be handled without tearing. It also permits one to obtain circular membrane samples of constant radius similar to those obtained with a hollow punch.

Egg yolk was isolated and washed in serum (0.9% NaCl) until albumen was completely eliminated. The vitelline membrane was isolated and cut into two hemispheric caps which were carefully washed in five successive serum baths to fully eliminate any trace of vitellus. One of the circular caps, used as the control specimen, was set on its holder and stored for 24 hr at +1°C, immersed in serum.

The other cap, set on an identical holder, was immersed in serum in a 300 mL cylindrical flask for freezing. In order to reduce concentration effects during freezing, the membrane was placed very near the flask bottom so that sample freezing started at the beginning of the freezing cycle when more than 90% of the serum was still in the liquid phase, thus minimizing ionic shock. The membrane was then frozen using a pre-determined set of freezing conditions, with the freezing interface crossing the membrane perpendicularly from its external to its internal side. Storage at −18°C for 16 hr was followed by a controlled thawing sequence in a +20°C atmosphere.

It should be noted that the present experiments mainly concern slow freezing and thawing cycles, i.e.: +20°C to −2.5°C for the first hour; −2.5°C to −5°C in the next 2.25 hr; −5°C to −18°C in the next 2 hr for the freezing sequence. The thawing operation was as follows: −18°C to −5°C for 0.5 hr; −5°C to −2.5°C in 2.5 hr; −2.5°C to +20°C in 3 hr.

Freezing equipment

The freezing equipment was a conventional cryostat, 30L volume, cooled through a double-stage Freon evaporator. With this equipment, controlled temperatures as low as −40°C, measured by a contact thermometer, can be maintained. We used −18°C for most of our experiments.

Temperature at the membrane level was measured with a plastic constantan-manganin thermocouple, 0.1 mm in diameter, 50 Ω in resistance. The sensitivity of this thermocouple was 38 μV/°C. It corresponded to a reading of less than 0.1°C with conventional measuring equipment consisting of a standard potentiometric recorder with a maximum sensitivity of 2.5 mV and a 25 cm chart width, which provided for a continuous recording of the temperature. The cold junction was placed in an ice-water mixture in a Dewar-flask.

Dehydration measuring equipment

To compare the drying rate of the initially water-saturated membranes, both were placed in a steady environment with constant T values of +20 ± 0.5°C and RH 55 ± 5%. The simultaneous drying of both control and frozen samples provided more accurate comparison since the effects of accidental variations of T and RH were

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counterbalanced. The drying rates were obtained from the water mass evaporation measurements provided by two 10^{-4}g scales (Mettler H 33) with measuring chambers which connect with each other and with the outside atmosphere (Fig. 1).

In order to extend the scope of the investigations, an infra-red analysis method provided another method for measuring the rate and extent of water desorption from the membrane. The principle of the method consists of measuring the variation with time of the percentage transmission of an infra-red beam directed through initially water-saturated frozen and control samples. Since I.R. transmission is a simple function of water concentration in the material under study, this method provided direct observations of the drying kinetics for the samples. The equipment used was a classical I.R. spectrophotometer (Beckmann IR 4220 type). Since it contained a built-in desiccation system, the measuring chamber was continuously swept out by completely dry air. This equipment then provides for: (1) a 0% relative humidity which tends to maximize the rate of dehydration of the membranes; (2) a 40°C drying temperature, which is close to the physiological incubation temperature and therefore prevents thermal destruction of the membrane.

In order to follow these dehydration phenomena, the 3490 cm^{-1} (wavelength 2.86 μm) was selected. This band corresponds to a very broad maximum in the I.R. spectrum of liquid water and provides for excellent accuracy in evaluating water concentration.

The method used for the provision of frozen and control membranes was identical to the preceding one. Membranes were subsequently stretched on "Irtran" frames (an I.R. transparent material, nonsoluble in water) and then water saturated at +40°C. After temperature and saturation equilibration, they were placed separately in the spectrograph chamber for drying. The percentage of transmitted light was then continuously recorded to measure directly the membrane drying kinetics.

RESULTS & DISCUSSION

Drying kinetics of membranes

From each desorption experiment, a series of double simultaneous results were obtained, i.e. those of control and frozen mass samples originating from the same vitelline membrane as a function of time.

In order to ascertain the reproducibility of these results and quantify them, about 50 similar experiments where carried out. Data from 13 of these experiments were retained on the basis of the following criteria: (1) the control and frozen samples originating from the same ovocyte did not show any tear and remained perfectly streatched in their holder to the end of the experiment; (2) the temperature and humidity conditions remained constant through the drying sequence.

Typical drying kinetics are presented in Fig. 2. The drying rates are obtained from the expression:

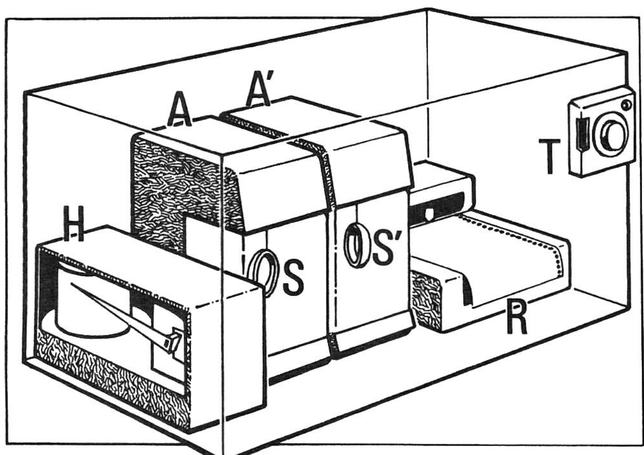

Fig. 1—Sketch of the drying test cabinet: A,A'-scales, S,S'-sample holders, T-clock, H-hygrometer, R-temperature recorder for thermocouples.

$$m = m_s + m_{H_2O} \quad (1)$$

where m_s is the asymtotic value of m, and m_{H_2O} the mass of evaporated water. Since the dry mass of membrane samples was not constant, the mean value of 13 pairs of measurements was used for kinetics characterization

$$\bar{m}_s = 2.0 \times 10^{-3}g \text{ and } \sigma = 0.6 \times 10^{-3}g \quad (2)$$

where $\bar{m}_s$ is the mean value and σ the standard deviation of the distribution. It is important to emphasize that the variations of m_s for a pair of samples originating from the same ovocyte were much narrower and did not exceed 4%. The variations in m_s, indicated by σ, can be explained by the structure of the external layer of the membrane whose thickness varied from 1 – 3 layers, depending on the egg concerned. At the same time, the water content of the thickest membrane was greater and, therefore, the drying period was longer.

In order to normalize all experiments, the parameters m^* and τ are introduced, where m^* is the ratio of water retained per dry mass of the membrane $m^* = (m-m_s)/m_s$, and τ the drying time of half the total mass of water.

Experimental data in terms of these parameters are therefore independent of m_s and τ, and are solely characteristic of the drying phenomena.

From Fig. 2, two results are immediately apparent: (1) the frozen membrane initially contained a much greater quantity of water than the controls after they were placed in identical water saturation conditions (i.e. after immersion in physiological serum); and (2) the dehydration rate of the frozen sample was greater than that of the control.

In addition, the frozen membranes were much more fragile than control (90% of samples which tore spontaneously during tests were frozen membranes). They often did not appear as clean and clear as the control membranes, and small gelatinous spots were observed in the frozen membranes.

Isothermic differential drying rate

In order to analyze more accurately these observations

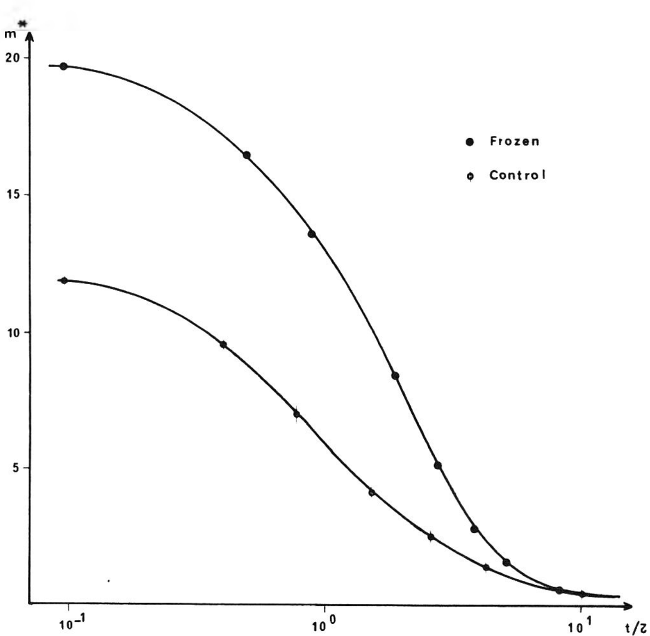

Fig. 2—Typical drying kinetics of frozen and control membrane samples. $m^* = (m-m_s)/m_s$ is the ratio of the moisture content in g_{H_2O} versus membrane dry mass in g and τ the dehydration time of half of the water amount. Experimental conditions: t = 20°C; RH = 55%.

and discuss the source of differences between control and frozen samples, another parameter was introduced, i.e. the isothermal drying ratio: $V = V_f/V_c$, where V_f and V_c are, respectively, the drying rate of the frozen and control samples at the same time, as derived from the preceding drying data. Thus for V_f at time $t = (t_1 + t_2)/2$:

$$V_f(t) = (m_{t2} - m_{t1})/m_s \times 1/(t_2 - t_1) \quad (3)$$

where m_{ti} is the mass of the frozen sample at time t_i, and m_s the mass of the same sample when dry, i.e. at $t = \infty$.

The curve thus obtained, $V = f(t)$, represents the variation of the drying rate of a frozen sample as compared to its control as a function of time at identical temperature and humidity conditions (Fig. 4).

This curve showed a very sharp maximum, which approximately corresponded to $V_f = 2.3\ V_c$. It followed from these results that the absorbed water in the frozen membrane showed very different properties from water in the control (Eisenberg and Kauzmann, 1969).

The curve presented in Fig. 3 shows the variation with time of $\overline{M}^* = f(t)$, i.e. the water mass ratio, m^*_f/m^*_c of the frozen and control membranes.

Some interesting results may be derived from Fig. 3. At the beginning of drying, $\overline{M}^* = 1.51$ which shows that, at membrane saturation equilibrium, frozen samples absorbed on an average 50% more water than the control and that, following a 10^3 sec initial drying period, during which M^* reached a maximum of 1.77, the frozen sample mass decreased less rapidly than the control sample mass.

Though $\overline{M}^*$ then decreased rapidly and regularly, the water mass of frozen sample was always greater than that of the control. Finally, at the end of drying, $\overline{M}^*$ became lower than 1 and reached 0.81.

Membrane freezing, therefore, has two important consequences which can be directly deduced from these results: (1) an average 50% increase in the initial water absorption capacity at saturation; (2) an average 20% decrease in water retention capacity at the end of drying.

During drying of frozen membranes initially saturated with water, the relative lost water balance was therefore about 70% at 20°C and 55% relative humidity.

The ratio of the mean dehydration rate of frozen membranes as compared to control, as a function of time, $\overline{V} = g(t)$ shown in Fig. 4, confirms the peak already observed. A detailed examination of the curve showed that the initially saturated frozen and control membranes have the same dehydration rate at the start of the experiment; the frozen samples then dehydrate much more rapidly, since at 10^3 sec the drying ratio more than doubled. The rate reached a maximum at 2.10^3 sec and at the end of the drying process, tended to fall back to 1. The relevant standard deviation was highest at the end of drying since $\overline{V}$ becomes a ratio of two figures, each tending toward zero.

This analysis therefore confirmed that freezing considerably changed the characteristics of water in membranes. The new behavior of water was characterized by a much increased drying rate and a marked tendancy for the membrane to become more dehydrated.

In the set of experiments carried out in the I.R. experimental set up, the readings obtained from frozen and control membrane samples were very similar to those obtained from weighing done at 55% R.H.

Comparison of the I.R.-measured drying kinetics for frozen and control membranes confirmed the conclusions reached in the first study and showed that, under completely different RH and temperature conditions, the observed features are the same.

At the start of the experiment, the water concentration W of the frozen membrane was far greater than that of control, since $W_f = 2.4\ W_c$. This confirmed the significant induced freezing increase in the membrane water absorption capacity already reported. During the course of drying, the frozen membrane dehydrated much more rapidly than its control and at the end, both membranes desorbed at the same rate and water concentration of the frozen and control membranes were roughly the same.

These results confirmed the tendency of frozen membranes to dry more rapidly than controls. The difference in the final degree of dehydration achieved was however not as significant as in the first gravimetric study. In the

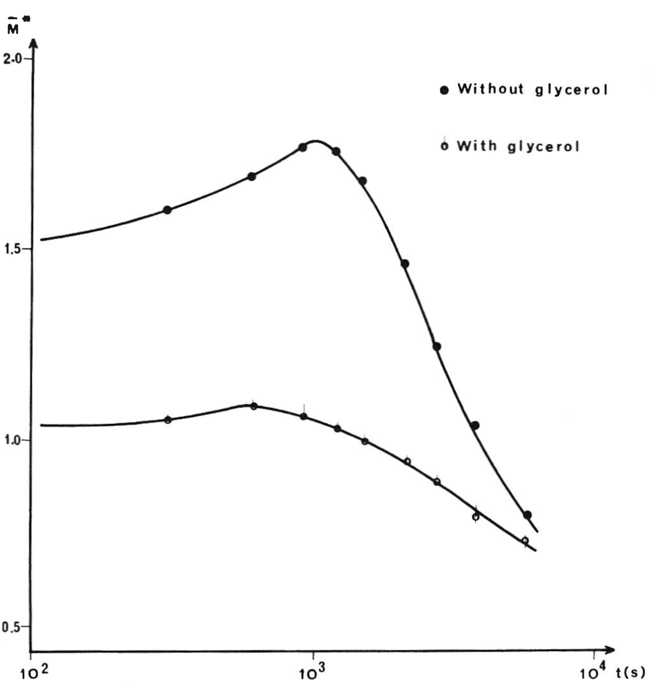

Fig. 3—Moisture content retained by frozen as compared to control samples $\overline{M} = m^*_f/m^*_c$ as function of time. Upper curve, normal test; lower, glycerol added.

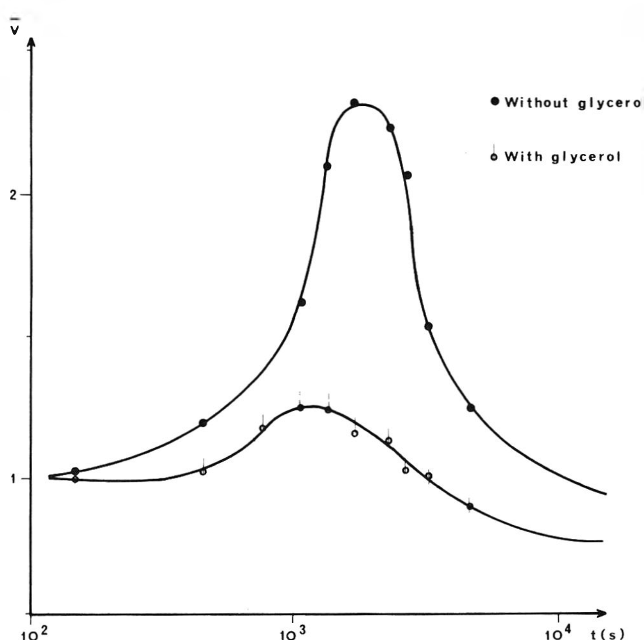

Fig. 4—Undimensional rate of moisture loss $\overline{V} = V_f/V_c$ as function of time. Upper curve, normal test, lower, glycerol added.

first study, the difference in the final dehydration was of the order of 20% because desorption was not pursued to an advanced stage, the RH being of the order of 55%, whereas it was 0% in the I.R. tests.

Cryoprotective action of glycerol

Glycerol was used in certain tests for two reasons (Doebbler, 1966). It is one of the oldest cryoprotectors known (Lovelock, 1953) and has been successfully used in cryobiology (erythrocytes, spermatozoa) (Rowe, 1968) (Rapatz and Luyet, 1968).

It is an intracellular protector for slow freezing conditions. The glycerol can therefore go through the vitelline membrane and eventually protect it from freezing injuries.

The experimental study was conducted in a manner similar to the preceding one.

Glycerol at 15% concentration was added to physiological serum baths for sample freezing and for control storage at +1°C.

Before taking any dehydration measurements, the glycerol was carefully washed out from samples by immersing them in five successive serum baths.

From the curve $M^* = f(t)$ (Fig. 3), it can be observed that glycerol treated and nontreated samples behaved differently after identical freezing-thawing cycles when they were submitted to the same water saturation and drying conditions.

When these results were compared with those from the preceding study of membranes without glycerol, it appeared from Fig. 3 that glycerol prevented the increase in water absorption capacity induced by freezing at water saturation equilibrium. It cancelled the increase with drying time of the retained water mass ratio between the frozen and control membranes and did not affect the tendency of frozen membrane to have a lower water content at the end of the drying process.

The influence of glycerol on the dehydration rate is clearly shown in Fig. 4 which compares the drying rate curve for glycerol and nonglycerol treated membranes as a function of time.

It is obvious that the significant increase in drying rate caused by freezing was strongly reduced by glycerol, V_{max} being reduced from 2.3 to 1.25. This slight maximum observed was most certainly due to the relatively low concentration of glycerol used (15%) as compared with those currently used in cryobiology (30 - 40%). This 15% concentration, in place of the usual 30% offers, however, the advantage of not requiring too long washing sessions, which increase the already strong probability of membrane tearing.

During the experiments with glycerol, a number of general results on biological tissues, which were already known, were observed; but, in addition, more specific phenomena relative to the vitelline membrane were demonstrated. It was shown that glycerol treated membranes have the same macroscopic appearance as the control, whereas as previously reported, without glycerol, their appearance was not so clean. They have a fragility equal to that of the control whereas absorption increases by 50% on untreated frozen membranes.

The experiments have shown that a freezing-thawing cycle strongly changed the initial water retention capacity, the drying rate and the final degree of dehydration of the vitelline membrane.

To interpret these phenomena a model has to be developed for the structure changes and denaturation produced by freezing, which takes into account already known data, and the new results acquired through this study.

One classical model that might support these results is the capillary model; but it also shows some inconsistency. It could be inferred from the analysis of the rate of moisture loss V versus moisture content MC in frozen and control membrane, Fig. 5, that the peak in V curve of Fig. 4 was mainly due to a prolongation of the "constant rate period" in the frozen membrane. The effective vapor diffusivity corresponding to the evaporation of free or slightly bound water as evaluated graphically from the Sherwood relation $M/M_o = A.e^{-BDT}$ in Fig. 6 is respectively $D_c = 1 \times 10^{-3}$ cm^2/sec^{-1} for the control and $D_f = 0.92 \times 10^{-3}$ cm^2/sec^{-1}. But the length of the "constant rate" period $L_c = 600$ sec for the control and $L_f = 1{,}200$ sec for the frozen sample is roughly proportional to the initial water content MC in Fig. 5: 13.5g_{H_2O}/g_{solid} for frozen and 7.6g_{H_2O}/g_{solid} for control.

The "falling rate" period starts with a nearly equal moisture content $MC_c = 4.25 g_{H_2O}/g_{solid}$ and $MC_f = 5 g_{H_2O}/g_{solid}$ but the diffusivity of vapor in frozen membranes is

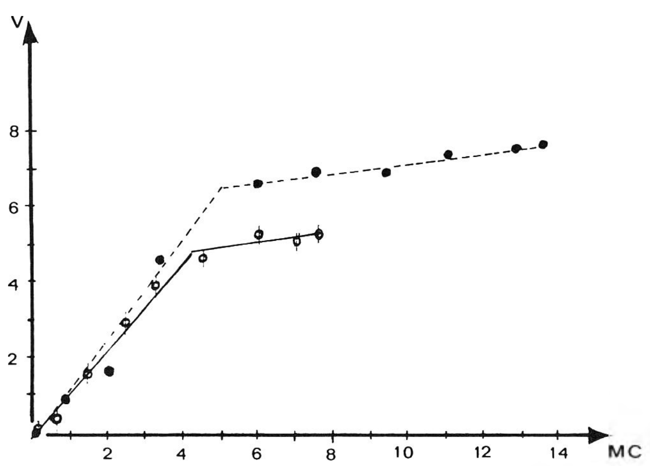

Fig. 5—Rate of moisture loss V in g_{H_2O}/g_{solid}/sec x 10^3 versus moisture content MC in g_{H_2O}/g_{solid}. The length of the "constant rate" period for frozen is about twice those for control as well as the initial MC. "Falling rate" period begins at roughly the same MC.

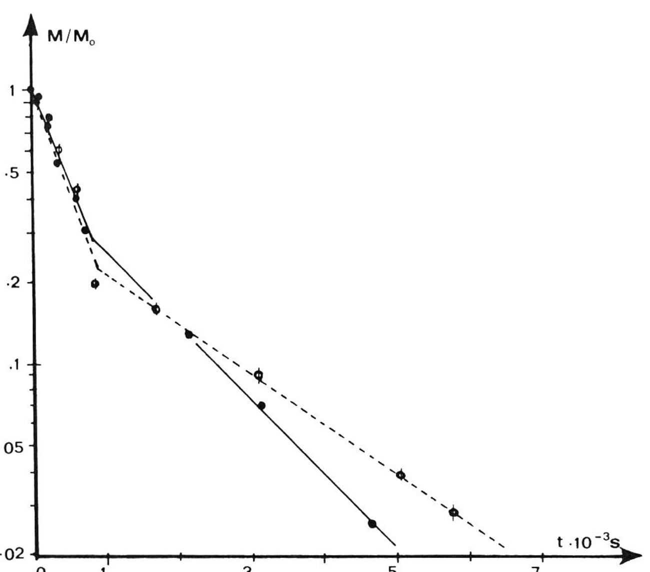

Fig. 6—Graphical evaluation of vapor diffusivity from Sherwood relation $M/M_o = A.e^{-BDT}$ where M/M_o is the MC vs initial MC_o, A and B constants depending on the material, D vapor diffusivity and t time.

larger than that for the control $D_f = 5.11 \times 10^{-4}$ cm²/sec^{-1} as $D_c = 4.26 \times 10^{-4}$ cm²/sec^{-1}, suggesting a more porous matrix in the frozen membrane, which supports the capillary model (Viaud and Aquire-Puente, 1972).

But the large increase of water retention capacity at saturation tends to support a model based on the formation of lamellar ice layers between constituent membrane layers. This model also supports the proportionality between "constant rate" period length and the amount of absorbed water in the samples.

We have already shown that glycerol prevented these modifications. It is a well known fact that glycerol, which is an intracellular protector in slow freezing conditions, acts by partly substituting itself for cellular water. In this particular study, where the biological material is a unique membrane, it is probable that glycerol also exchanged with part of the membrane water and thus prevented formation of ice inside the membrane.

Conversely, if no cryoprotector is used, ice crystals can grow inside the membrane itself, fed by free or slightly bound water, and consequently destabilize the membrane structure through inter alia a "loosening" of its constitutive layers: the maximum effect being presumably felt in the most hydrophobic areas of this heterogeneous multilayer system.

CONCLUSION

IT HAS BEEN POSSIBLE to suggest, through the gravimetric method of differential isothermic dehydration, a structural, or at least physiological, denaturation of the vitelline membrane submitted to a freezing-thawing cycle, even if it keeps its macroscopic physical integrity.

As the state of the membrane does condition the nature and the rate of dehydration in ambient conditions, it has been shown that freezing entailed irreversible changes in the vitelline membrane, indicated by: a significant increase in the absorption capacity; an accelerated drying rate; and a marked decrease in the final water retention capacity.

The results were confirmed by dehydration with the infra-red method.

Deep disturbances occurred in the properties and/or structure of the thawed vitelline membrane as compared with the control.

These modifications could have been demonstrated on a multi-layer membrane because it provided a noticeable amount of water for amplification of the phenomena, which are the more significant, the greater the number of layers in the membrane. It is quite obvious that on a thinner type membrane which is only two layers thick, for instance, such effects could not have been measured through these tests and are to be checked by other methods if they really occur.

Finally, it has been shown that glycerol, which is known to be an intra-cellular protector in slow freezing conditions also happens to be a surface or membrane protector in such conditions. Through the use of glycerol, most of the modifications observed on the vitelline membrane and induced by freezing can indeed be avoided.

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A Model for Mechanical Degradation of Wheat Starch in a Single-Screw Extruder

V. J. DAVIDSON, D. PATON, L. L. DIOSADY, and L. J. RUBIN

ABSTRACT

The effect of extrusion cooking in a single screw extruder on the molecular weight distribution of wheat starch was correlated to the starch melt rheology. A simple first-order model has been developed which defines the extent of mechanical degradation of the amylopectin component as a function of nominal shear stress and residence time in the extruder. A limited set of experimental observations were used to test the model. Iso-shear stress curves were calculated for the experimental range of conditions. The curves can be used, in conjunction with estimates of the residence time, to calculate equivalent processing conditions, and to predict the extent of amylopectin degradation. The model may allow the use of small extruders to determine fundamental process parameters for full scale extrusion.

INTRODUCTION

EXTRUSION COOKING is widely used to modify functional properties, such as paste viscosity, water solubility and water absorption of cereal flours and starches. Although these physical properties are important in specific product applications, it is necessary to define the extent of modification at a molecular level in order to understand and control the extrusion process. Studies conducted by Charbonniere et al. (1973) and by Mercier and Feillet (1975) have shown that at low extrusion temperatures (70 - 95°C) the crystal structure of starch is disorganized and that starch is solubilized in macromolecular forms. Colonna and Mercier (1982) recently reported on an investigation of structural modifications of manioc starch by processing in a twin-screw extruder. Extrusion cooking produced starches of lower intrinsic viscosities than the unprocessed material, and gel permeation chromatography showed that starch polymers were degraded into macromolecular components. Colonna and Mercier (1982) further established that the size distributions of the β-limit dextrins of the extrudates varied considerably, pointing to degradation of the amylopectin component.

The macromolecular nature of the breakdown products of starch degradation has surprised researchers and no one has attempted to explain the underlying mechanism producing these changes. Recent studies of the structural changes in wheat starch processed in a single-screw extruder (Davidson, 1983) indicated that mechanical stresses were an important factor in the degradation of the amylopectin fraction. The purpose of this paper is to discuss the nature of mechanical degradation, the development of a simple, first-order model for starch degradation, and the application of this model to the experimental observations which have been presented in an earlier paper (Davidson et al., 1983).

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Polymer degradation by mechanical forces

The subject of mechanochemistry of polymers has been reviewed by Baramboim (1964) and Casale and Porter (1971). In the molten state, polymers exhibit apparent viscous flow behavior. However, a macromolecular fluid with a broad distribution of molecular weights, may contain large chains of high characteristic viscosity at the processing temperatures. Also, temporary entanglements may form and significant stresses can develop in localized portions of the molecular chains leading to scission. The rupture sites are generally at branch points, central portions of chains and bonds between hetero-atoms. The largest molecules are most susceptible to mechanical degradation effects and the reaction products are macromolecular.

Mechanical degradation reactions of polymers have been shown to be first order (Baramboim, 1964; Basedow et al., 1979). Basedow et al. (1979) also reported that shear stress was the controlling factor in the degradation process and that the rate constant was a function of shear stress. Temperature and molecular weight affect mechanical degradation essentially by their contribution to viscosity. As the molecular weight increases, so does the melt viscosity and therefore the shear stress at equivalent shear rates. Mechanical degradation decreases with temperature up to a maximum temperature. The negative temperature coefficient reflects the effect of temperature on viscosity and is a critical factor in a mechanochemical reaction (Casale and Porter, 1971). At higher temperatures thermal and oxidative degradation can occur.

Due to the overlapping of different degradation mechanisms, it is difficult to separate mechanical, oxidative and thermal effects in tests that simulate processing conditions. Also there is a strong feedback effect on the kinetics because as the degradation proceeds, the physical properties of the material change considerably. In the present work, a simple model has been developed to describe the mechanical degradation of the amylopectin in the extruder channel.

MATERIALS & METHODS

Materials

At 19 mm diameter, single-screw extruder powered by a 2.2 kW SCR controlled motor was used throughout these experiments. The extruder was similar to the C.W. Brabender 2003 extruder but modified to allow the barrel to be opened along its horizontal axis to expose the screw (Paton et al., 1980). The inside surface of the barrel was rifled with eight grooves running parallel to the screw axis.

The flow geometry was kept constant during this study. A single flight, uniform pitch screw with a 3:1 compression ratio, a feed depth of 0.38 cm and a blunt tip was used in all cases. The zone at the end of the screw had a volume of 12.7 cm^3. A 6.3 mm (i.d.) circular die was used.

All experiments were conducted using Whetstar 4 (lot #101723J), a commercial wheat starch donated by Industrial Grain Products, Montreal, Canada.

Methods

The following operating conditions were varied in a 3 × 2 × 2 factorial design: (a) barrel temperature, 121°C, 149°C, 177°C;

(b) screw speed, 50 and 100 rpm. and (c) feed moisture, 20 and 25%. The desired moisture level was achieved by spraying distilled water as a fine mist onto starch in a Patterson V-blender. The extruder was operated at steady state for each set of conditions. Attainment of steady state was judged by a constant operating torque and by a regular extrusion rate. At these operating conditions, the melt quickly reached the temperature of the barrel well and remained isothermal (Davidson, 1983). At the end of an experiment, the barrel was quickly split open to examine the screw contents and to determine the axial length over which the physical transition of the starch from solid to melt occurred.

Residence time distributions were measured at each level of the operating parameters using MnO_2 as a tracer (Davidson et al., 1983).

The structural characteristics of the extrudates were analyzed by gel permeation chromatography, enzymatic digestion and dilute solution viscometry (Davidson et al., 1984).

Four of the 12 experiments were duplicated. Based on the similarities of the operating torques and residence time distributions for duplicate processing conditions as well as the similarity of paste viscosity curves of duplicate products, repeatability was considered satisfactory. Due to the time required for each chromatographic analysis (30 hr), only the 12 samples representing different experimental levels were analyzed in duplicate on a Bio-Gel 150M column.

RESULTS & DISCUSSION

Mechanical degradation model

The data used in developing the mechanical degradation model are given in Tables 1 and 2. These tables summarize the gel permeation analyses presented in a recent publication (Davidson et al., 1984) which indicated that wheat starch was significantly degraded during extrusion procssing. The disappearance of the largest molecular species was strongly affected by factors related to shear stress. In order to develop a model to relate the extent of degradation to extruder operating variables, several simplifying assumptions were made.

First, it was assumed that no mechanically induced degradation occurred in the section of the extruder filled with solid starch but was limited to material in a melted state, as in the solid state mechanical degradation usually results from impact rather than shear forces (Casale and Porter, 1971). Secondly, it was recognized that in some cases the degradation was due to a combination of mechanical and thermal factors which would be difficult to separate. For purely mechanical degradation, it was assumed that shear stress was the critical factor. However, the analysis of shear stresses in the extruder channel was a complex problem due to: the existence of two-phase flow in the transition region; the two-dimensional flow characteristics of the fully melted zone; and the rheological nature of the starch melt.

The effect of the transition zone was neglected in the initial analysis and only the fully melted region was considered. Due to the high operating temperatures in this study, the phase transition occurred over a relatively small volume in the extruder: usually the transition zone was less than 15% of the total volume of the partially and fully melted zones. Hence the residence time of fluid material in the transition zone was short relative to the time spent in the fully melted zone.

Middleman (1977) suggested that the nominal shear rate [V_{bz}/H defined in Eq. (1)], is a reasonable estimate of the deformation rate experienced by the fluid. Average nominal shear rates over the fully melted length of the extrusion channel were calculated using the experimental records made after the barrel was split open. For the extruder geometry and operating conditions used in these experiments, the nominal shear rates were approximately 30 s^{-1} at 50 rpm and 60 s^{-1} at 100 rpm. These were an order of magnitude higher than the average shear rates calculated for material in the die section. Thus it was assumed that mechanical degradation was occurring primarily in the fully melted starch along the length of the screw channel.

The characterization of the rheological nature of the melt was a difficult problem. Most rheological studies have not considered the effects of high shear rates or the shear rate history that the material experiences during the rheological measurements. Some mechanical degradation may occur during testing thus changing the molecular weight distribution and the rheological characteristics. In the

Table 1—Characteristics of the wheat starch in the fully melted zone of the extruder channel

Process variables	Nominal shear rate (s^{-1})	Melt viscosity (10^3 Nsm^{-2})	Nominal shear stress (10^4 Nm^{-2})	Mean residence time (s)	τt (10^5 Nsm^{-2})	x_{exp}
50 rpm: 20% moisture						
121°C	32.1	1.7	5.4	5.0	2.7	0.66
149°C	26.5	1.2	3.2	15.2	4.9	0.57
177°C	26.5	0.8	2.2	17.0	3.8	0.53
100 rpm: 20% moisture						
121°C	56.3	1.3	7.2	5.6	4.0	0.57
149°C	50.8	0.9	4.5	10.6	4.8	0.43
177°C	53.2	0.6	3.2	9.6	3.0	0.53

Table 2—Characteristics of wheat starch in the fully melted zone of the extruder channel

Process variables	Nominal shear rate (s^{-1})	Melt viscosity (10^3 Nsm^{-2})	Nominal shear stress (10^4 Nm^{-2})	Mean residence time (s)	τt (10^5 Nsm^{-2})	x_{exp}
50 rpm: 20% moisture						
121°C	28.6	1.2	3.4	14.1	4.8	0.76
149°C	28.1	0.8	2.2	13.6	3.0	0.59
177°C	26.5	0.6	1.5	18.0	2.7	0.62
100 rpm: 20% moisture						
121°C	53.8	8.8	4.7	11.7	5.5	0.38
149°C	58.1	5.6	3.2	8.7	2.8	0.62
177°C	52.1	4.0	2.1	13.0	2.8	0.57

absence of definitive studies of these effects, the power law viscosity model suggested by Harper et al. (1971) was used to calculate the melt viscosity:

$$\eta = 78.5 \exp(2500/T)\exp(-7.9M)\dot{\gamma}^{-0.49} \quad (1)$$

where η = apparent viscosity; T = temperature, °K; M = moisture fraction; $\dot{\gamma}$ = nominal shear rate V_{bz}/H, s^{-1}; V_{bz} = down-channel component of tangential velocity of barrel surface relative to the screw; and H = depth of extrusion channel.

Although this model was not derived from wheat starch, it seemed to be the most appropriate of the few viscosity models for extruded food materials that have been reported in the literature. The viscosity model of Harper et al. (1971) was adequate for predicting the effects of varying moisture, temperature and screw speed on nominal shear stress. When the nominal shear stress was used in combination with the residence time data, the severity of processing could be assessed for different operating conditions.

The extent of molecular degradation had to be characterized in order to develop a kinetic model for the mechanical degradation. Since the unprocessed wheat starch contained polymers with a wide range of molecular weights and sizes, it was difficult to define a simple parameter that would reflect the extent of modification and that was consistent with the information contained in the analytical data. Since the largest molecules are most susceptible to mechanical degradation, the relative change in weight fractions of carbohydrate eluted in the void volume of the Bio-Gel 150 M column ($K_a \leqslant 0.04$) was used to characterize the extent of mechanical degradation. (Davidson et al., 1984). The term χ_{exp} was introduced to denote the fraction of large molecular weight material remaining in the extrudate as measured experimentally:

$$\chi_{exp} = \frac{\text{Amount of extruded sample eluted in void volume}}{\text{Amount of unprocessed starch eluted in void volume}} \quad (2)$$

Hence the fraction $(1-\chi_{exp})$ was the estimate of the extent of degradation of the amylopectin component of starch.

Smaller molecular species may also be degraded by mechanical forces but it is impossible to differentiate intact molecules from degradation products based on the chromatographic information.

The intrinsic viscosity represented an average molecular size and also showed the degradation effects of extrusion processing. However, it was affected by other degradation reactions such as thermal mechanisms which change the overall molecular size distribution so it could not be used to define the extent of purely mechanical degradation.

Basedow et al. (1979) used a viscometer with a well defined shear field to show that shear stress was the controlling factor of the degradation process and the rate constant was an exponential function of shear stress. Since it was not simple to define the shear conditions in the extruder channel, the nominal shear rate in the down-channel direction was used to characterize the deformation rate experienced by the melt. Also, it was not possible to vary the residence time for controlled shear stress conditions in the experiments reported in this work but the combination of shear stress and residence time was known for each of the extrusion conditions. Since the range of shear stresses was small, the "rate constant" function was modified to a linear form:

$$k = k'\bar{\tau} \quad (3)$$

where k' = modified constant; and τ = mean shear stress defined at the nominal shear rate, V_{bz}/H, and the barrel temperature.

Using the parameters, χ_{exp} and k, defined for this study, the following rate expression was proposed for the mechanical degradation of wheat starch in the extruder:

$$\chi_{exp} = e^{-kt} \quad (4)$$

where t = mean residence time of the melt in the screw channel.

The mean residence time of the melt in the screw channel was calculated as a fraction of the average residence time of the active volume, t_a (Davidson et al., 1983). Since the mass flowrate was constant through the extruder, the fraction of the overall average residence time was calculated as:

$$t = t_a \times \frac{\text{Mass hold-up in fully melted zone of screw}}{\text{Total mass hold-up}} \quad (5)$$

Application of model to experimental data

The chromatographic data were plotted in Fig. 1 against the product of residence time and shear stress calculated using the rheological model of Harper et al. (1971). Ten of the twelve experimental points were used to fit the parameters of the following equation by linear regression:

$$\ln \chi_{exp} = -k'(\tau t) + b \quad (6)$$

The correlation coefficient was −0.93 and the parameter estimates were: $k' = 1.7 \times 10^{-6}\ m^2/Ns$, and b = −0.05.

Two experimental observations were well removed from the fitted regression line. In the one case (149°C/50 rpm/20% moisture), the residence time measurement was suspect. A duplicate run was made later in the experimental program and the records indicated a shorter length of fully melted material in the screw which would translate into a shorter residence time. This was the only set of

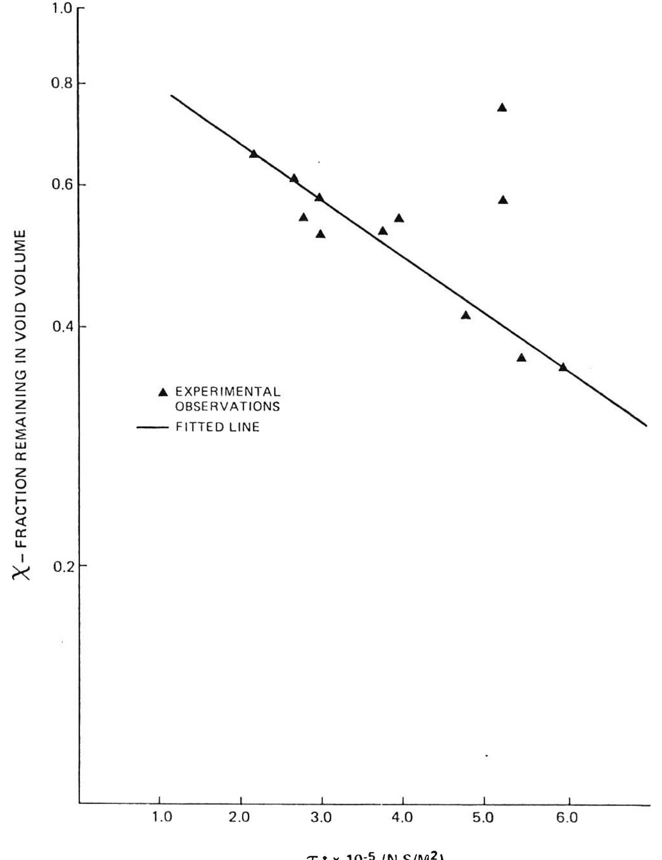

Fig. 1—Fitted regression line for mechanical degradation model.

duplicates where the lengths of the transition zones were quite different and in fact, this may reflect an error in the experimental records. The lowest extent of change in the void-volume material was observed at operating conditions of 121°C and 25% moisture, and this experimental point did not lie on the regression line. Since it was expected that the rheological properties of the melt depended on the extent of reaction, the viscosity predicted by the power law model may not be valid in this range.

The proposed model seems to explain the observed degradation of the amylopectin fraction of wheat starch in the extruder. The changes in this fraction likely have a strong effect on the functional properties of the extrudate; thus it may not be possible to interpret some of the product characteristics in terms of the operating variables.

The model does not attempt to explain some of the subtle change in the distribution of molecular sizes observed in the chromatograms of the extruded products (Davidson et al., 1984). These were probably due to the superposition of thermal stresses on mechanical effects which result in degradation of molecular species smaller than the void volume fraction. It was impossible to quantitatively separate the two effects based on the experimental data that were available.

The strong interaction of barrel temperature and moisture effects on the characteristics of the extrudate has been reported in the literature (Lawton et al., 1972; Paton and Spratt, 1978). It has been observed that more than one combination of these two operating conditions produce extruded starches with similar properties. These observations can now be explained on the basis of the proposed degradation model, and a strategy for predicting equivalent operating points has been developed in the present study. Using the rheological parameters of Harper et al. (1971) to describe the effects of temperature and moisture at a standard shear rate (1 s^{-1}), iso-shear stress curves were calculated for a range of conditions comparable to the experimental conditions. These are plotted in Fig. 2. It was apparent from following along the 62.8 N m^{-2} curve that the standard shear stress was the same at 25% moisture and 120°C as at 20% moisture and 147°C. Since the actual shear stress varies according to the nth power of the shear rate (where n is less than 1), the effect of changing screw speed on the shear stress can be estimated using the standard shear stress and the appropriate nominal shear rates. This information, in conjunction with estimates of the residence time, can be used to calculate the severity of processing conditions and hence to predict the extent of degradation of amylopectin. A laboratory-scale extruder could be used to define the ranges of shear stress and residence time that effect certain structural modifications since small-scale experiments would offer advantages in cost and time. These experiments would provide fundamental information that would be useful for scale-up to larger extruders.

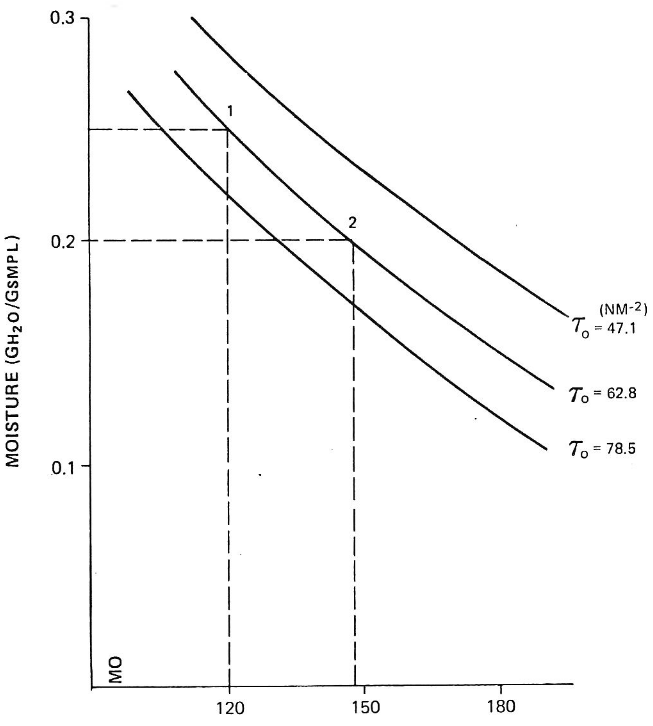

Fig. 2—Iso-shear stress curves predicted for wheat starch for the experimental extrusion conditions.

CONCLUSIONS

THE CHANGES in starch chemistry which occur during extrusion cooking are closely linked to the melt rheology in the extruder. The proposed, first-order model is useful as a means of predicting the extent of mechanical degradation of the amylopectin component of starch. Experimental data for extrudates with different degrees of structural modification were used to verify the model.

An iso-shear stress diagram was developed from rheological data to predict equivalent operating points in terms of barrel temperature, feed moisture and screw speed. It is suggested that this information could be used to judge the severity of processing conditions and hence to estimate the extent of degradation of amylopectin in large-scale extruders.

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Comparison of Four Procedures of Cheese Meltability Evaluation

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ABSTRACT

Two traditional cheese meltability tests, the Schreiber and Arnott, as well as two microwave modifications thereof were compared on a variety of mild and sharp cheddar, process American, and mozzarella cheeses and various process cheese products. There was a marked lack of correlation between the Schreiber and Arnott results indicating that at least two material properties control meltability. The effects of variations in heating time and oven temperature in the nonmicrowave tests and heating time in the microwave tests were determined. Differential scanning calorimetry did not reveal differences useful in predicting melting behavior.

INTRODUCTION

IN MANY CHEESES, notibly mozzarella, cheddar, and process American, melting characteristics are prime factors in the determination of quality for particular product applications. Methods for objective assessment of the melting and resolidification patterns have therefore, become much needed by both the dairy industry and the variety of other industries utilizing melted cheese in their products (e.g. pizzas and prepared frozen meals).

Conceptually, the main problem in objective determination of the attributes commonly referred to within "meltability" is that they are related to both the heat transfer and thermal phase change characteristics of the solid cheese and to the rheological or flow properties of the melt. Although both are determined by the cheese composition and microstructure, their study is complicated by the fact that they are highly interdependent and transient properties. A further difficulty in objective meltability assessment is that in a melting cheese specimen there are always temperature gradients that are determined not only by the cheese thermal properties but also by the external temperature distribution, the oven relative humidity, and the geometry of the system. Since temperature differences may also be reflected in phase or structural changes and since the geometry of the system drastically changes in time, the application of the standard assumptions for thermal analysis may be inappropriate.

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From the rheologist's point of view, the situation is just as difficult because the stress distributions are ill-defined and the specimen shape is hard to control. Under such circumstances, it appears that there is a clear advantage to the use of simple empirical methods and such methods are indeed employed by the industries that produce or utilize melted cheese. The most commonly reported methods for meltability assessment were described by Schreiber (Kosikowski, 1977) and Arnott et al. (1957). Both methods are based on heating a standardized cylindrical cheese specimen under specified conditions (oven temperature and time) followed by measuring the specimen's height decrease (Arnott) or its diameter expansion (Schreiber). Since both procedures have not been standardized, different researchers have used various specimen dimensions and heating conditions they found most suitable (e.g. Breene et al., 1964; Keller et al., 1974; Schafer and Olson, 1975; Kovacs and Igoe, 1976; Chang, 1976; Sood and Kosikowski, 1979; Rayan et al., 1980; Hokes et al., 1982). They also defined different meltability indices derived from either the time to reach apparent meltability or the dimensional changes that were observed in the specimen. Comparison of the different indices of meltability and the conditions under which they were obtained is given in Table 1. Other attempts to define meltability in more rigorous physical terms also ran into difficulty. Lee et al. (1978) for example, tried to determine meltability by recording the temperature at which flowability became measurable by a Brookfield viscometer. Since the maximum torque that can be measured by the instrument is principally determined by construction considerations and since heat transfer within the sample could not be accurately monitored, the meltability indices so measured cannot be free from the influence of arbitrary factors. When a capillary rheometer was used to determine the flow curves of melted cheese (Smith et al., 1980), it was revealed that, with few

Table 1—Cheese meltability indices and the conditions under which they were obtained

Cheese type	Approximate specimens Diam (mm)	Approximate specimens Height (mm)	Heat source	Heating medium temp (°C)	Heating time (min)	Meltability index	Reference
Process cheddar	17	17	Oven (air)	100	15	Percent decrease in height	Arnott et al. (1957)
Cheddar	17	17	Water bath	80	—	Time required for melting (sec.)	Weik et al. (1958)
Cheese spread	30	20	Forced draft oven	110	8	Distance of flow from reference line (mm)	Olson and Price (1958)
Pizza	15	5	Water bath	98	5	Percent decrease in height & increase in diam	Breene et al. (1964)
Process	19	6	Oven (air)	232	3	Percent increase in diameter	Chang (1976)
Cheese spread	38	5	Oven (air)	204	5	Diam increase by flow line number	Kovacs and Igoe (1976)
Process	41	5	Oven (air)	232	5	Diam increase by flow line number (Schreiber)	Kosikowski (1977)
Process	38	8	Oven (air)	250 + 100	5 + 15	Percent increase in diameter	Sood and Kosikowski (1979)
Caseinate curd	25 (ball)	—	Microwave oven	—	0.25	Melt area per unit wt (cm^2 g^{-1}	Hokes et al. (1982)

exceptions, slippage and strong viscoelastic effects can make the results inapplicable.

Recognizing the fact that meltability is regulated by a multitude of factors and that it is a response to the external conditions imposed on the cheese, this work was undertaken to quantify the effects of these conditions, particularly temperature history, on the empirical meltability parameters.

MATERIALS & METHODS

Materials

Mild and sharp cheddar, mozzarella, process cheese products, and process American cheese from different manufacturers were purchased at a local supermarket. The samples were stored for 24 hr at 4°C prior to testing.

Arnott test

Cylindrical samples with controlled dimensions were prepared by a wire cutting device shown in Fig. 1. After preparation, the specimens were wrapped again and stored at 4°C until testing. The specimens (17 × 17 mm) were placed on a glass Petri dish and placed in a 100°C laboratory oven for 15 min (except where noted otherwise). The center height of each specimen was measured immediately after removal from the oven by a tripod micrometer. The percent length decrease was calculated and is reported as the Arnott meltability on a scale of 0–100.

Schreiber test

Specimens (length, 4.8 mm; diam, 41 mm) were placed in an oven preheated to 232°C for 5 min (unless otherwise noted). The specimens were then removed and cooled for 30 min at room temperature. Specimen

Table 2—Meltability indices of various commercial cheese samples bought from a supermarket

		Schreiber test								Arnott test	
Type	Manufacturer	A	B	C	D	E	F	Mean measurement	Mean	Measurement	Mean
Sharp Cheddar	Kraft	7	5	7	8	8	7	7.0		69	
		5	4	5	10	10	9	7.2	7.1	65	67
		7	5	6.5	9	8	7	7.1		68	
	Land O'Lakes	6	5	6.5	10	8	9	7.4		74	
		6	5	6	10	8	6.5	6.9	7.2	72	73
		6	5	6	10	8	8	7.2		73	
	Stop & Shop	7	5	5	7	10	10	7.3		67	
		8	5	10	10	8	7	8.0	7.9	71	70
		10	8	6	8	8	10	8.3		71	
	Pricechopper	10	9	6	6	5.5	7	7.3		85	
		9	8	7	7	6	7	7.3	7.3	82	82
		9	8.5	6	7	6	6.5	7.2		80	
	Hood	7	7	7	7	8	8	7.3		72	
		7	7	7	10	9	6	7.7	7.5	72	73
		7	7	6.5	10	9	6	7.6		72	
Process American	Kraft	7	7	8	7	7	7	7.2		68	
		6.5	7	7	7.5	8	8	7.3	7.3	72	69
		8	6	7	7	7.5	8	7.3		67	
	Land O'Lakes	4.5	6	5	4	4	4	4.6		69	
		4.5	4	4	5	5	5	4.6	4.7	70	71
		5	4	6	5	5	4	4.8		74	
	Stop & Shop	4.5	5	5	6	6	5	5.3		68	
		5	4	5	6	6	5.5	5.3	5.3	77	72
		5	3.5	5	5.5	7	6	5.3		70	
	Borden	4	4	4.5	5	5.5	5	4.7		80	
		4	4	5	5	4	5	4.5	4.6	74	78
		5	5	5	4	5	4	4.7		80	
Mozzarella	Kraft	4	4	4	4	3.5	3	3.8		74	
		4	4	4	3.5	3.5	4	3.8	3.8	72	74
		4	4	4	3	3.5	4	3.8		76	
	Axelrod	6.5	6	4	5	4	4	4.9		25	
		4	4	4	4	4	3.5	3.9	4.4	27	26
		5	5	4	4.5	4	4	4.4		25	
	Pricechopper	7	6	5.5	6	6.5	5	6.0		74	
		6	6	5.5	6	6	5	5.8	6.0	72	73
		7	6	6	6	6.5	6	6.3		74	
Process Cheese Product (Imitation & Diet cheeses)	Hoffman's	4	4	4	4	4	4	4		41	
		5	3	3	4	5	5	4.2	4.3	49	41
		5	4	4	5	5	4	4.6		33	
	Hood	4	2	4	4	3	5	3.7		2	
		3	5	6	4	5	3	4.3	4.3	6	7
		5	5	4	6	5	5	5		13	
	Kraft	7	7	8	6.5	2	4.5	5.8		66	
		6	8	8	7	4.5	4.5	6.3	6.1	56	60
		6	6.5	7	6	5	5	6.1		59	
	Clearfield	6	6.5	7	7	6.5	7.5	6.8		44	
		6.5	5.5	6	7.5	9	7	6.9	7.0	37	38
		6.5	5.5	7	7.5	8	9	7.3		33	
	Borden	10	8	10	6.5	6.5	8	8.2		7	
		10	10	7	8	6	7	8.0	8.1	10	8
		10	9	9	6.5	6	8	8.1		8	
	Weight Watchers	10	10	4	6	10	10	8.3		23	
		10	10	8	6.5	8	10	8.8	8.5	14	18
		10	10	7	6.5	8	9	8.4		16	
	Sun Glory	10	10	10	10	10	10	10		37	
		10	10	10	10	10	10	10	10	28	32
		10	10	10	10	10	10	10		31	

expansion was measured along six lines (A-E) marked on a concentric set of circles (Fig. 2) as described by Kosikowski (1977). Schreiber meltability is given as the mean of the six readings on the arbitrary scale of 0–10 units.

Microwave oven tests

The Arnott and Schreiber tests were also performed with the substitution of the conventional laboratory oven with a microwave oven (Radarange, Touchmatic II, Amana, Inc.) and shortening the heating times to 0–60 sec. The meltability indices are reported in the same manner as for the conventional oven tests.

Repetition

Each meltability test under given conditions was performed on 3–4 fresh specimens. The results shown are the mean values of these tests.

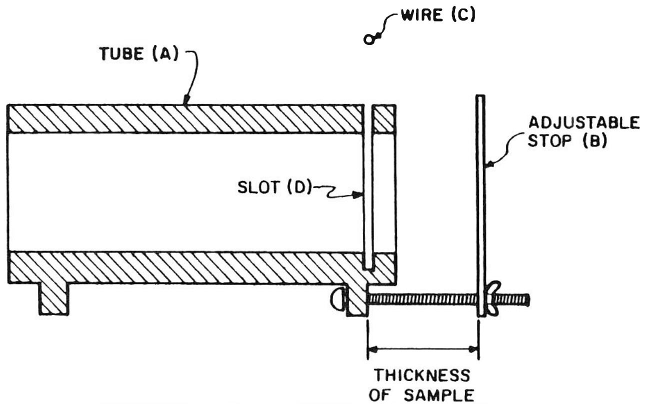


Fig. 1—Cutting device for producing samples of uniform thickness from a plug of cheese.

Differential scanning calorimetry (DSC)

Thermograms, i.e. heat flow vs temperature relationships, of samples of American, mozzarella, and cheddar cheese in the range 10–70°C were obtained using a Perkin-Elmer Model DSC-2 differential scanning calorimeter. The sample weights were 20–25 mg. They were placed in a volatile sample holder and heated at 5°C per minute. The sensitivity of

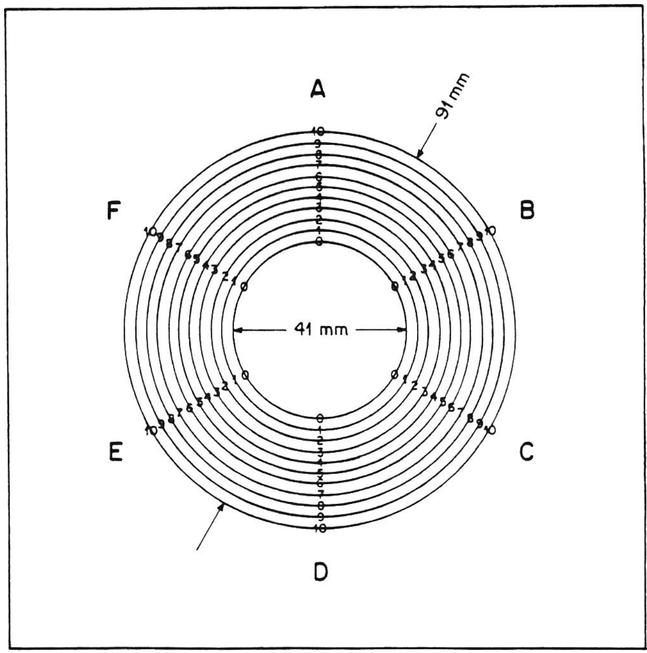
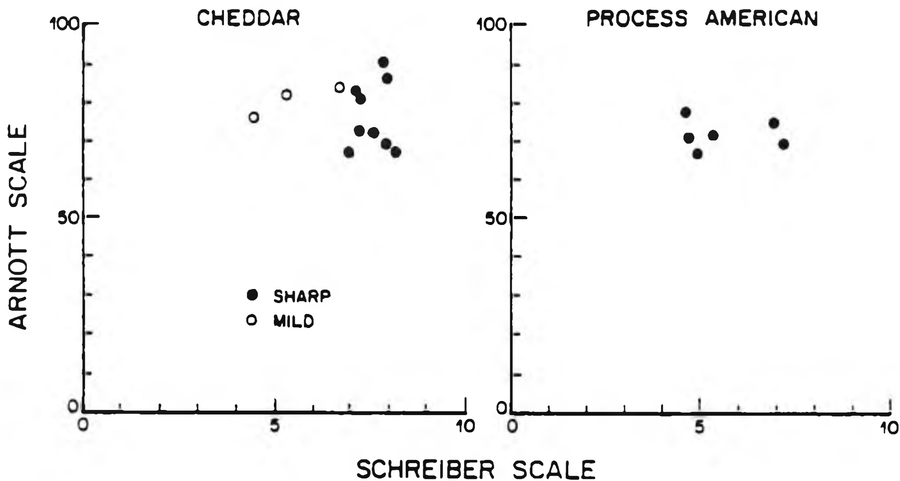


Fig. 2—Measuring device used in Schreiber test.



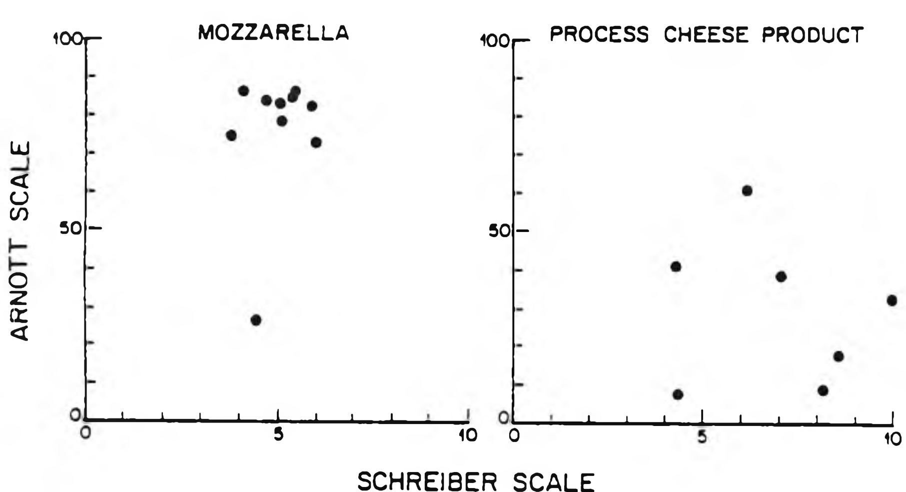


Fig. 3—Arnott vs Schreiber test meltability scores.

the instrument was set in the range 2–20 mcal per sec and the recorder chart speed to 2 cm per min.

RESULTS & DISCUSSION

CONSIDERING THE NATURE of the tests, both the Arnott (100°C, 15 min) and Schreiber (232°C, 5 min) methods provided consistent and reproducible results (Table 2). Theoretically, if the melting specimens had maintained their volume and cylindrical shape, and if the heat effects had been the same in both tests, the two meltability indices should have been interrelated. This, however, was not the case as can clearly be seen in Fig. 3 (which also contains additional data not reported in Table 2). The lack of correlation between the two meltability indices indicates that heating had different effects on different kinds of cheeses, which was confirmed by the general shape of the melting specimen and its alteration in time (Fig. 4). It appears that, because of the different structural properties of the cheeses, the rheological characteristics of the melts were different which gave rise to shape distortions that were partly a reflection of the flowability differences between the fully melted and unmelted regions within the specimen.

Effect of heating time and oven temperature

As previously mentioned, it is difficult to give a quantitative account of the physical changes that occur in melting cheese specimens. Qualitatively, however, the time-temperature-flow relationships ought to be considered in light of the following aspects. Higher oven temperatures or longer heating times will reduce temperature variations within the sample roughly in a manner that corresponds to conductive heating. They can also, however, depending on the hot air humidity, increase evaporative processes and the formation of a film on the surface of the specimens, both of which will effect heat transfer to the specimens thus altering their internal, transient temperature distributions. The meltability indices previously described, being single parameters, account for both flow (or deformation) before and after all the specimen material is melted. Thus, if the relative contribution of the two stages is different in different cheeses, such indices do not measure the same set of properties in them. Furthermore, the Arnott and Schreiber tests are performed at temperatures that are considerably different from each other. It is expected that the melt consistency

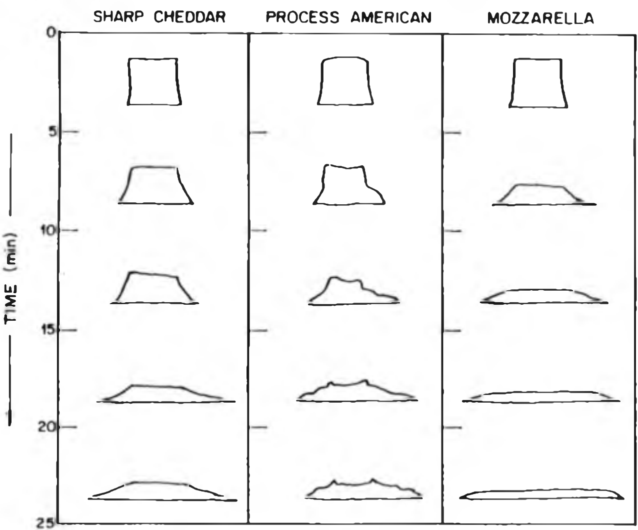


Fig. 4—Typical shape changes of cylindrical cheese samples (oven temperature = 100°C).

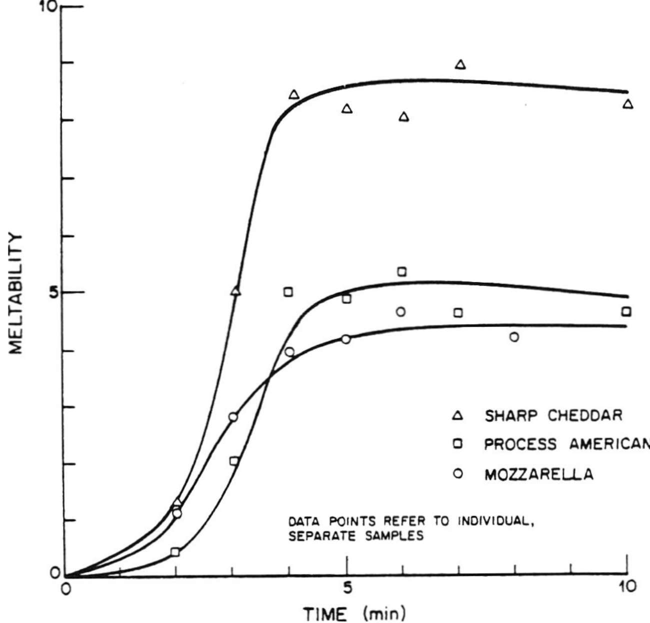


Fig. 5—Schreiber test meltability vs heating time.

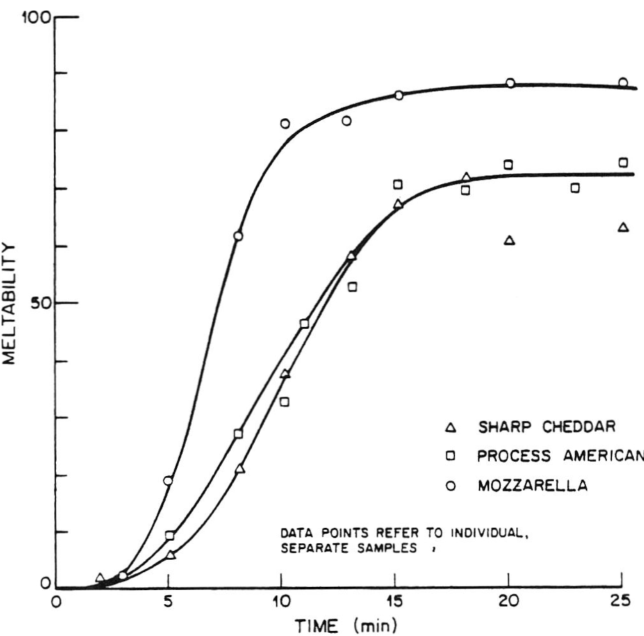


Fig. 6—Arnott test meltability vs heating time.

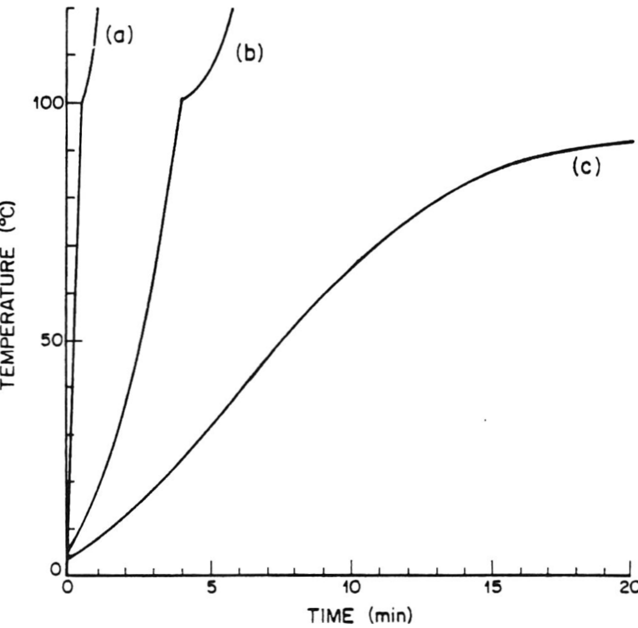


Fig. 7—Specimen center temperature vs heating time in (a) microwave Arnott, (b) conventional Schreiber, and (c) conventional Arnott tests.

and its temperature dependency will be characteristic of each cheese and again it can be concluded that the two tests do not measure the same rheological attributes even if the effects of other factors (e.g. heat transfer coefficients) could be ignored. Because of these considerations, it was of interest to study how the two indices were affected by the exposure time and whether the temporal relationship was a cheese characteristic.

Examples of such relations are presented in Fig. 5 and 6. These demonstrate that the 5 and 15 min durations in the Schreiber and Arnott tests (respectively) were both in the plateau regions of the curves which means that, after this time, little or no further changes in cheese dimensions are expected. The absolute rates to reach these plateau values among the cheeses and between the tests primarily because of temperature differentials (Fig. 7).

Microwave oven tests

Results of the microwave oven experiments are presented in Fig. 8 and 9. In contrast with the conventional oven experiments,

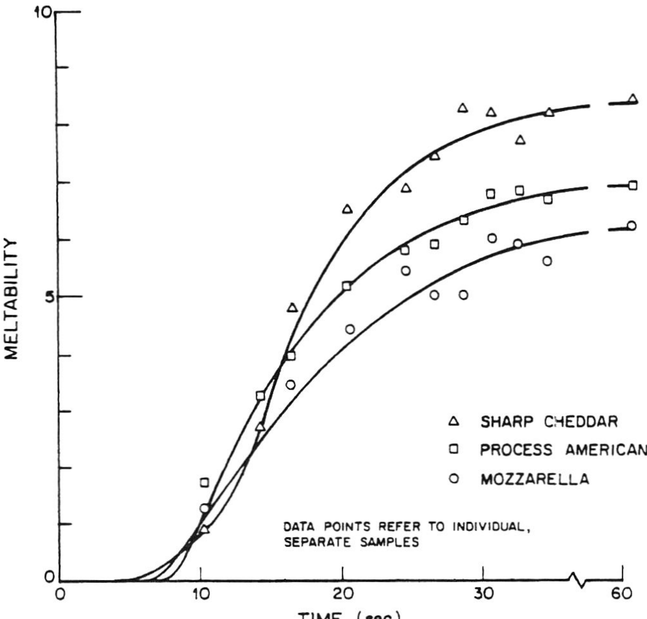


Fig. 8—Microwave Schreiber meltability vs heating time.

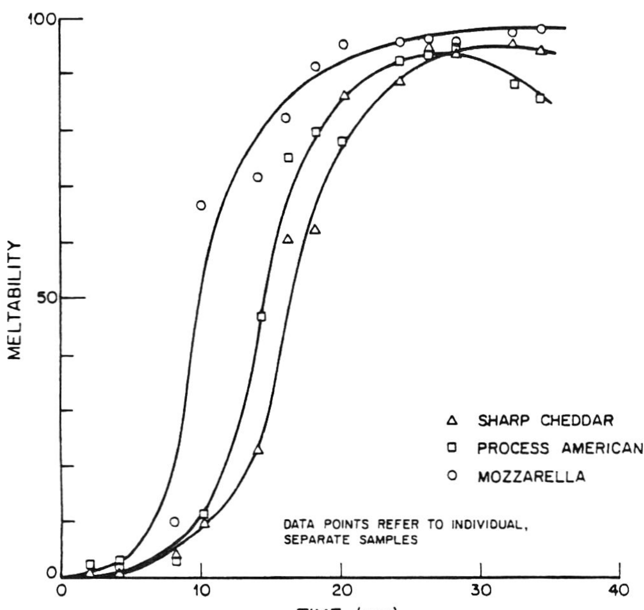


Fig. 9—Microwave Arnott meltability vs heating time.

the time scale was quite short. Also, the temperature distribution within the specimen was mainly regulated by factors other than heat transfer to the surface and therefore it can be assumed that the specimens were totally melted on a time scale of 20–60 sec rather than 5–15 min of the former tests. Again, however, no clear correlation could be found between the two meltability indices. In this case, however, the results mainly reflect the test geometry and the melt's rheological properties and, to a lesser extent, the effect of the transient stage prior to full melting. It can therefore be concluded that in any meltability evaluation, the rheological and thermal aspects ought to be considered as equally important and no single parameter can meaningfully account for both.

Differential scanning calorimetry

Thermograms of American, mozzarella, and cheddar cheese are shown in Fig. 10. Although slight differences can be detected, they do not appear to indicate major differences in the meltability patterns. In these tests, unlike those in the convection, or even the microwave ovens, transient heat transfer effects were negligible because of the small specimen sizes used. The interpretation of these thermograms indicates, therefore, that the differences in meltability of these three cheeses are associated with differences at very low energy levels — well below those of latent or even sensible heat. This is in accord with the widely known and obseerved fact that meltability properties are primarily determined by the microstructural and rheological characteristics of the cheese and only to a lesser extent by its gross composition.

It appears, therefore, that well defined meltability criteria ought to be based on a comprehensive rheological analysis presenting the data in terms of temperature and time dependent parameters. Such rheological information will have to include elasticity (Yang and Taranto, 1982) as well as the more conventional flow parameters. The measurement of such parameters experimentally, however, still remains a formidable task because of still unsolved problems regarding fat separation during the test. Until this challenge is met and the rheological methods developed, it seems advisable to treat the currently used parameters as relatively crude indicators which only distinguish large melting differences rather than as quantitative meltability criteria.

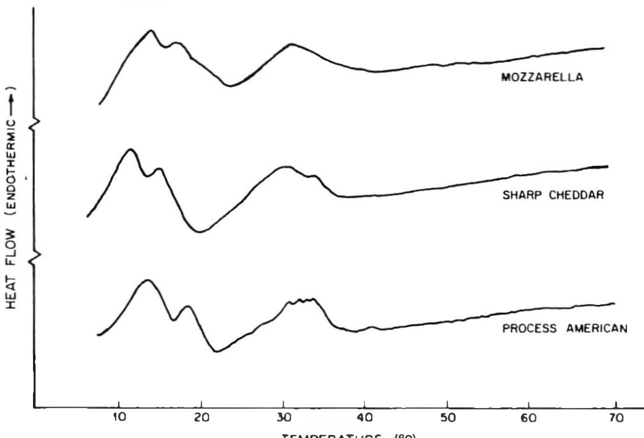


Fig. 10—DSC thermogram of three cheese samples in the range 4–70°C (single samples).

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—Continued on page 1170

Effect of Cooking Temperature and Animal Age on the Shear Properties of Beef and Buffalo Meat

J. ROBERTSON, D. RATCLIFF, P. E. BOUTON, P. V. HARRIS, and W. R. SHORTHOSE

ABSTRACT

Semimembranosus muscle samples from young (24 - 29 months) and old (48 - 54 months) buffalo and Brahman-Shorthorn steers of similar carcass weights, within age groups, were heated for 1 hr at 11 temperatures from 40 - 95°C. Warner-Bratzler initial yield force values indicated that myofibrillar strength was not affected by animal age or species. For stretched muscles, (where effects due to differences in myofibrillar contraction state would be minimal), peak force and peak force minus initial yield force values were greater (a) for older animals only when cooking temperatures were >60°C and (b) for buffalo than for beef over the 40 - 95°C range. These results suggested that there were species differences in the mechanical properties of undenatured as well as partially denatured connective tissue.

INTRODUCTION

THERE HAS BEEN little work reported on the quality of meat from the water buffalo (*Bubalus bubalis*) even though it is one of the more numerous of the world's domesticated animals (Ragab et al., 1966; El Koussy et al., 1977; Calub et al., 1971; Arganosa et al., 1973; Joksimovic and Ognjanovic, 1977; Robertson et al., 1983). The most recent work (Robertson et al., 1983) compared the properties of muscles from buffalo and cattle within the same carcass weight and age range grown together under normal pasture and good husbandry conditions in the Northern Territory of Australia. This work indicated that connective tissue made a bigger contribution to toughness for buffalo than it did for beef.

In other studies (Bouton and Harris, 1972; 1981; Bouton et al., 1974; 1981) the effect of cooking temperature and time on the shear properties of beef from animals of widely different age groups has been investigated. The results of these studies strongly supported earlier work (Machlik and Draudt, 1963; Draudt et al., 1964; 1971) which suggested that the decrease in shear force values, which occurred as the cooking temperature was varied from 50 - 65°C, was related to changes in the collagenous connective tissue. It was also shown (Bouton et al., 1974; 1981) that both the magnitude and the direction (i.e. increase or decrease) of the change in shear force values, obtained for meat samples cooked at temperatures between 50 and 65°C, was dependent on myofibrillar contraction state as well as animal age.

The work described inthe present paper was designed to investigate the effect on the mechanical properties of muscle, from buffalo and beef animals, of myofibrillar contraction state, animal age and cooking temperatures in the range 40 - 95°C. This temperature range encompassed major changes (Davey and Gilbert, 1974) in both the connective tissue, since collagen shrinks at 62 - 68°C and denatures at higher temperatures, and myofibrillar proteins, since myosin denaturation is essentially complete by 70°C. The prime objective was to determine whether previously (Robertson et al; 1983) detected differences in the mechanical properties of muscles from buffalo and beef animals of similar age were affected by animal age and/or cooking temperature.

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MATERIALS & METHODS

Animals, animal treatments

A total of 16 buffalo and 16 Brahman-Shorthorn steers were used. Eight of each species group were aged 48 - 54 months while the other 8 were aged 24 - 29 months. All the animals were fasted for 24 hr and weighed before being transported to a nearby abattoir where they were held overnight in pens with water available. They were slaughtered about 2 days after being taken off pasture.

After slaughter the carcasses were split and both sides hung by the Achilles tendon. Within 1 hr of slaughter one side from each carcass was rehung from the sacrosciatic ligament (i.e. tenderstretched) while the other side was left hanging from its Achilles tendon allowing some muscles to shorten during chilling. All sides were chilled for 48 hr at 0 - 2°C before the semimembranosus (SM) muscles were removed, trimmed of extraneous fat, placed in polyethylene bags and then frozen in cartons at about −20°C for a minimum of 1 wk. After at least 1 wk the cartons were taken under refrigeration from the abattoir to the laboratory where they were stored at −32°C. Samples were thawed, as required, in a chiller at 0 - 1°C for 48 hr on wire racks.

Cooking methods and muscle treatment

Each SM muscle was trimmed at each end by transverse cuts. The remaining muscle was then divided into 12 sub-samples of 100 - 120g, i.e. rectangular blocks measuring about 8 x 4 x 4 cm with the muscle fibers parallel to the longest side. These sub-samples were then assigned at random to each of the treatments, namely raw or cooked at 40, 45, 50, 55, 60, 65, 70, 75, 80, 87.5 or 95°C for 1 hr. They were cooked in close fitting polyethylene bags totally immersed in water baths controlled (± 0.5°C) at the appropriate temperature. After cooking, the samples were cooled in cold running water, placed in a polyethylene bag and stored overnight at 0 - 1°C. After storage these samples were then prepared for Warner Bratzler (WB) shear force measurements.

Sarcomere length and pH measurements

A light diffraction method (Bouton et al., 1973) was used to measure sarcomere lengths on the post rigor raw samples. A Townson expanded scale pH meter with a Phillips (C64/1) probe type combined electrode was used to measure ultimate pH values directly on the raw muscle samples at room temperature (c. 22°C).

Shear force measurements

The WB shear force device has been described in detail previously (Bouton and Harris, 1972). The samples for the device were rectangular strips, usually 3 - 5 cm in length and with a 1 cm^2 cross-section (0.67 x 1.5 cm) with the fibers lying parallel to the longest length. The parameters measured from the WB shear force deformation curves were (a) peak force, i.e. the maximum force recorded and (b) initial yield force, i.e. the force at which sample first began to yield.

Statistical methods

Analysis of variance was used to test for the significance of treatments effects and to calculate, where appropriate, standard errors and, hence, least significant difference (LSD) values between means at the $P < 0.05$ level.

RESULTS

THE AVERAGE fasted liveweight of the Buffalo and the Brahman-Shorthorn steers was not significantly different within either the group of young (297.0 and 298.6 kg, respectively) or the group of old (432.0 and 438.9 kg respectively) animals. The pH measurments showed no significant differences due to either animal age or to species and all the muscles used had an ultimate pH between 5.4 and 5.6.

Sarcomere lengths obtained for the raw SM muscles showed no significant differences due to animal age but those from tenderstretched beef sides were significantly longer than those from tenderstretched buffalo sides. The mean sarcomere lengths (± S.D.) obtained, over the two age groups, for the buffalo and beef, respectively, were 1.75 ± 0.02) and 1.78 ± 0.03 μm for the muscles from the Achilles tendon hung sides and 2.68 ± 0.02 and 2.84 ± 0.02 μm for those from the tenderstretched sides (the LSD for comparison of means was 0.09). These sarcomere length results showed that the muscles from the Achilles tendon hung sides had shortened, albeit not severely, and tenderstretching had produced a wide difference in the myofibrillar contraction state between the muscles from the Achilles tendon and sacrosciatic ligament hung sides.

Results obtained for muscles from Achilles tendon hung sides

The WB shear force results obtained for the muscles from the Achilles tendon hung sides are shown in Fig. 1 and 2. The results have been plotted to show the effect of (a) animal age and (b) species. Initial yield force values increased steadily with increased cooking temperature up to about 70°C with no significant animal age or species differences. At temperatures above 70°C the initial yield force values tended to decrease with increased temperature. The peak shear force values also increased steadily with increas-

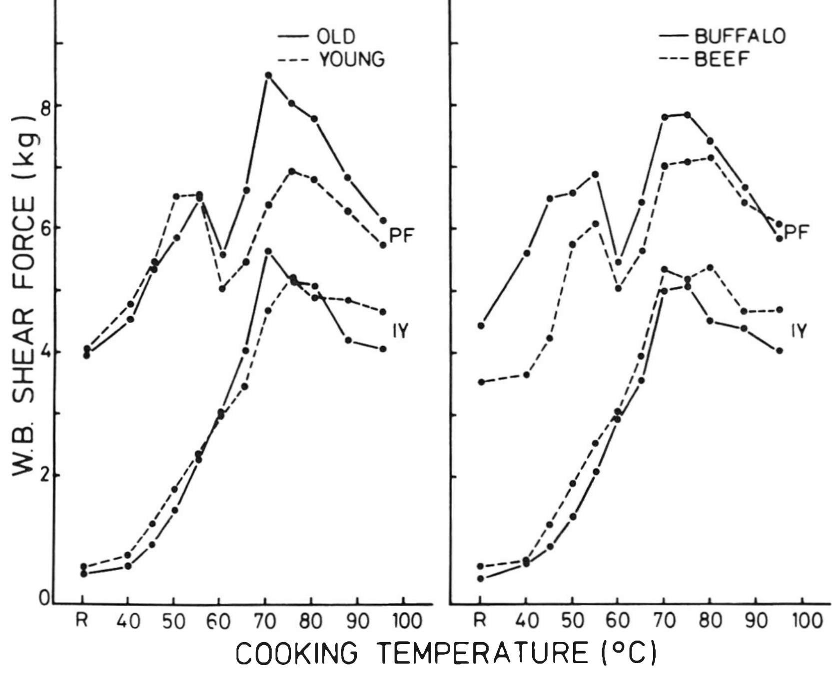

Fig. 1—WB peak shear force (PF) and initial yield (IY) force values obtained for SM muscles, from Achilles tendon hung sides of buffalo and beef cattle groups aged 24 - 29 (young) and 48 - 54 months (old), raw (R) and cooked at various temperatures for 1 hr. LSD values for peak force means were 1.11 kg for between species or age and 1.03 kg for within species or age comparisons. For initial yield force means the corresponding LSD values were 0.94 and 0.77 kg, respectively.

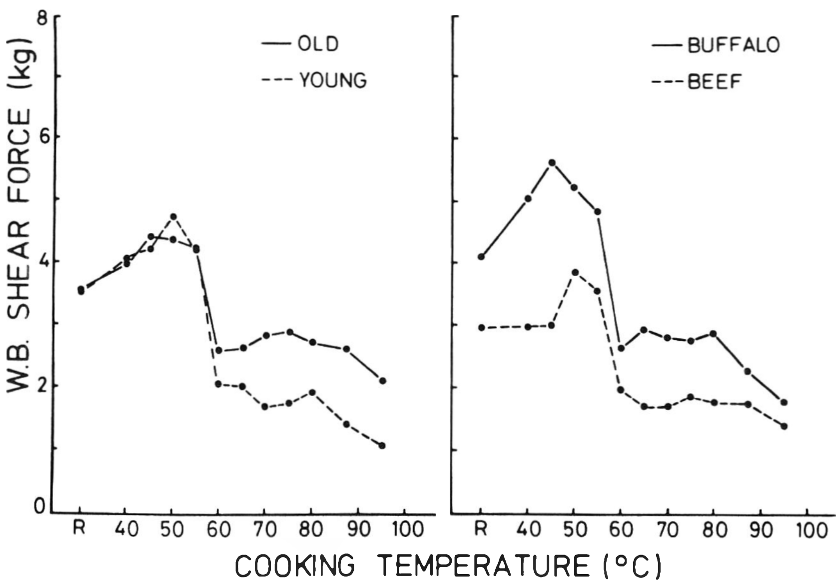

Fig. 2—WB peak shear force minus initial yield force means obtained for SM muscles, from Achilles tendon hung sides of buffalo and beef groups aged 24 - 29 (young) and 48 - 54 months (old), raw (R) and cooked for 1 hr at various temperatures. LSD values were 0.83 for between species or age and 0.77 kg for within species or age comparisons.

ing temperature but between 55 and 60°C there was a sharp decrease. As the temperature was increased above 60°C the peak shear force values continued to increase with increasing temperature to reach a maximum between 70 and 80°C. Peak force values then tended to decrease with temperature greater than 70 - 80°C.

In Fig. 2 it can be seen that peak minus initial yield force values tend to increase with increase in temperature up to 45 - 50°C then to decrease between 55 and 60°C. Above 60°C changes with increasing temperature were small until at 75 - 80°C shear values generally decreased. At temperatures below 60°C there was no significant animal age effect; buffalo, however, had significantly higher values than beef.

Results obtained for stretched muscles

The shear force results obtained for the buffalo and beef muscles from the tenderstretched sides have been summarized in Fig. 3 and 4. The mean initial yield force values (Fig. 3) for both animal age and both species increased with increasing cooking temperatures up to 80°C after which they decreased. There was no significant effect

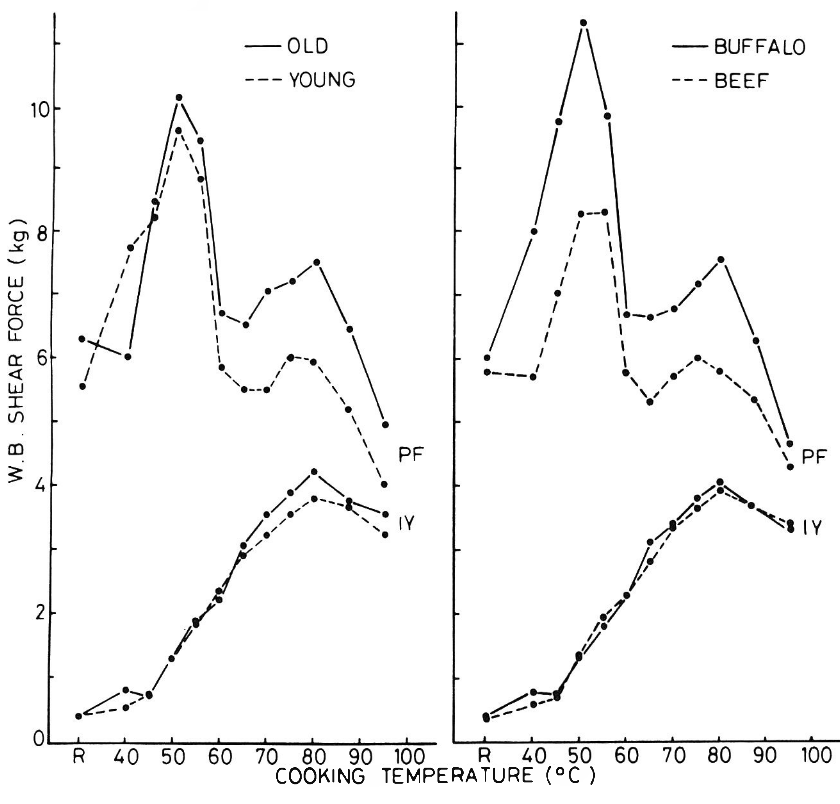

Fig. 3—WB peak shear force (PF) and initial yield (IY) force means obtained for stretched SM muscles from buffalo and beef cattle groups aged 24 - 29 (young) and 48 - 54 months (old), raw (R) and cooked at various temperatures for 1 hr. LSD values for peak shear force means were 1.09 kg for between species or age and 1.05 kg for within species or age comparisons. For initial yield force means the corresponding LSD values were 0.37 and 0.33 kg, respectively.

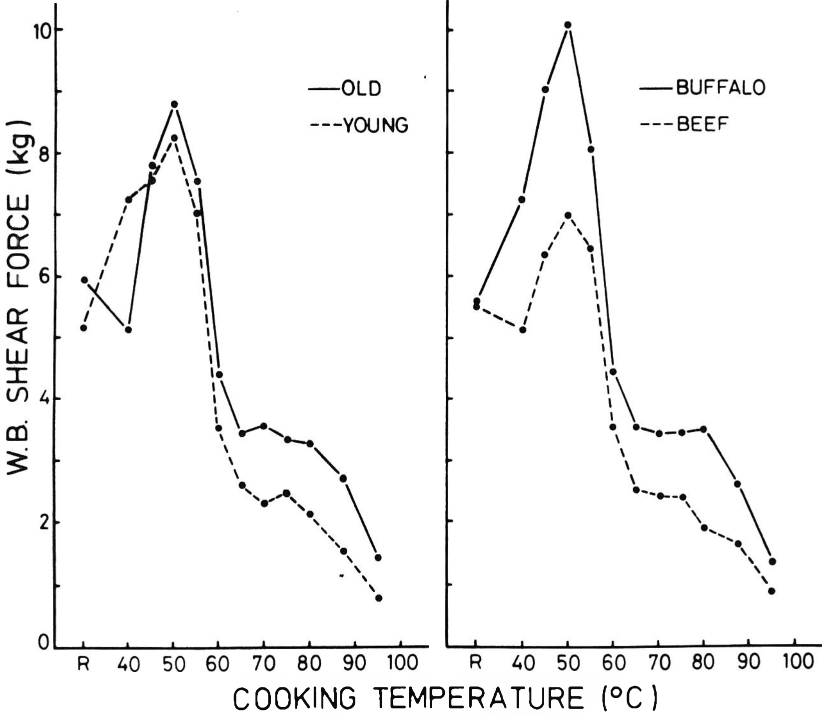

Fig. 4—WB peak shear force minus initial yield force means obtained for stretched SM muscles, from buffalo and beef groups aged 24 - 29 (young) and 48 - 54 months (old), raw (R) and cooked at various temperatures for 1 hr. LSD values were 1.02 for between species or age comparisons and 0.97 for within species or age comparisons.

due to either animal age or species in these initial yield force results. The peak shear force values increased with cooking temperatures up to about 50°C but decreased rapidly and significantly with a further decrease above 55°C reaching a minimum at 60 - 65°C. At high temperatures (>65°C) peak shear force shear values increased again to reach a maximum at 75 - 80°C. For temperatures above this peak, shear force values decreased again with an increase in temperature. These results compared well with results obtained earlier (Bouton et al., 1981) which used stretched SM muscles from beef animals of widely different ages (0 - 3 months to 8 - 15 years). In this earlier work there was little difference due to animal age with cooking temperatures up to 50°C and the biggest effect attributable to animal age occurred at temperatures >55°C'

Peak force minus initial yield force values, which are believed to give an indication of the connective tissue contribution to peak shear force values (Bouton et al., 1975, 1977), also increased with cooking temperature up to about 50°C then decreased rapidly with increase in temperature except for a levelling off at temperatures between about 75 and 80°C (Fig. 4). Values decreased again with further increase in temperatures above 80°C. The peak force minus initial yield force values were significantly greater for the older animals at all temperatures >65°C except 95°C. In the present study the buffalo had consistently greater values than the equivalent beef samples over the entire 40 - 95°C range of cooking temperatures.

DISCUSSION

PREVIOUS INTERPRETATIONS of shear force deformation curves (Bouton et al., 1975; 1977) were that initial yield force values were a relative measure of the contribution of the myofibrillar structure to the strength of cooked meat. In the present experiments the inital yield force results indicate that the strength of the myofibrillar structure in SM muscles from young or old buffalo and young or old beef animals does not differ significantly at particular cooking temperatures over a wide (40 - 95°C) temperature range for muscles in similar contraction states. This lack of effect of animal age on initial yield force values contrasts with that of a previous report (Bouton et al., 1981). They found that SM muscles from tenderstretched carcasses of 8-15 years old beef cows had significantly greater initial yield force values than those from tenderstretched carcasses of young (0 - 3 months old) beef calves. The age difference (0 - 3 months vs 8 - 15 years) was, however, far greater than used inthe present experiments (24 - 29 months vs 48 - 54 months).

The marked decrease in Warner-Bratzler peak shear force values that occurs with increase of the cooking temperature from 50 - 65°C has been the subject of considerable research (Machlik and Draudt, 1963; Tuomy et al., 1963; Draudt et al., 1964; Schmidt and Parrish, 1971; Bouton and Harris, 1972, 1981; Draudt, 1972; Bouton et al., 1981). Both the magnitude and direction of this change has been shown in beef muscles (Bouton and Harris, 1972, 1981; Bouton et al., 1981) to be highly dependent on animal age. The nature of the changes suggested that temperature-induced effects on connective tissue were involved. Using stretched ST muscles, Bouton and Harris (1981) found that peak shear force values decreased as cooking temperatures increased above 50°C in veal (0 - 3 months old), above 55°C for 2 - 4 year old cattle but did not decrease at all for muscles from very old cows (8 - 15 years old) when using a 1 hr cooking period.

This evidence (Bouton and Harris, 1981) together with all the other evidence (Machlik and Draudt, 1963; Tuomy et al., 1963; Draudt et al., 1964; Schmidt and Parrish, 1971; Bouton and Harris, 1972; Draudt, 1972; Bouton et al., 1981) indicated that connective tissue changes were involved. The results in Fig. 3 (and to a lesser extent Fig. 1) indicated that animal age (24 - 29 months vs 48 - 54 months) had little effect on the mean peak shear force values of beef or buffalo until cooking temperatures used were above 60°C. However, for the stretched muscles, the peak shear force values obtained for the buffalo samples were significantly greater than those obtained for beef over most of the 40 - 95°C range of cooking temperatures.

It has been argued (Bouton et al., 1975; 1977) that the differences between peak shear force and initial yield force values are a relative measure of the connective tissue contribution to peak shear force values. Such differences could represent changes in the elastic characteristics of the connective tissue due to denaturation, animal age, or to restraint or to combinations of these factors. For both shortened (FIg. 2) and stretched muscle (Fig. 4) cooked at temperature up to 50 - 55°C there was virtually no effect of animal age on peak force minus initial yield force values. Above 60°C, however, there was a consistent age effect. There was a large and consistent difference due to species (the buffalo having the greater values) over the 40 - 95°C range of temperatures.

It would appear that after exposure to cooking temperatures well below the denaturation temperatures of beef collagen (62 - 68°C; Davey and Gilbert, 1974) the connective tissue in buffalo has different mechanical properties from that in beef. Whether this difference is due to molecular differences between beef and buffalo collagen, or ground substance, or to differences in the architectural distribution of the connective tissue through the muscle obviously requires further investigation.

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True and Apparent Retention of Nutrients in Hamburger Patties Made from Beef or Beef Extended with Three Different Soy Proteins

C. W. MILES, J. ZIYAD, C. E. BODWELL, and P. D. STEELE

ABSTRACT

Hamburger patties containing all beef or beef extended (20% reconstituted soy product, 80% beef) with soy isolate, soy concentrate or textured soy flour, or beef extended with one of the three soy products fortified with iron (60 mg/100g soy protein) and zinc (25 mg/100g soy protein), were analyzed in both the raw and cooked states for moisture, protein, fat, Ca, P, Mg, Na, Fe, Cu, Zn, and Mn. The percent true nutrient retentions (% TR) and percent apparent nutrient retentions (% AR) were calculated. The % TR was not significantly different for any of the patties for protein, fat, total ash, Ca, or Cu. The % TR of moisture decreased as the refinement of the soy added to the patties increased. The % AR was higher than the % TR for all nutrients examined.

INTRODUCTION

SOY PRODUCTS are becoming increasingly important in the human diet as economical sources of high-quality protein with valuable functional properties (Coppock, 1974). Ali et al. (1982) state that the primary advantages of soy products as meat extenders are increased yield with reduced cooking losses and lower cost without substantially lower nutritive value. Extension of meat products with soy protein is becoming more common than in previous years. Hamburger extended with soy protein is widely used in the military and in the USDA School Lunch Program.

Various processing methods produce different soy proteins. Hexane-extracted soy flakes which contain about 50% protein are ground into soy flour. The soluble carbohydrates are removed from the soy flakes to make a concentrate with 70% protein. Both soluble carbohydrates and fiber are removed from the soy flakes to make soy isolates with 90% protein.

Wolf (1970) noted that one of the functional properties of soy protein, its hydrophilic nature, allows it to absorb and retain water. Numerous investigators have found increased cooking yield and decreased cooking loss in ground beef products extended with soy proteins (Anderson and Lind, 1975; Drake et al., 1975; McWatters, 1977; Ali et al., 1982; Shaner and Baldwin, 1979; Bowers and Engler, 1975; Nielsen and Carlin, 1974; Ziprin and Carlin, 1976; Carlin et al., 1978; Ziprin et al., 1981; Williams and Zabik, 1975). Moisture retention rather than fat binding was largely responsible for the increased juiciness and cooking yield of beef patties extended with soy protein (Anderson and Lind, 1975; Drake et al., 1975; McWatters, 1977). Drake et al. (1975) explained that raw ground beef patties extended with textured soy protein (TSP) contain more moisture than do all-beef patties, possibly because the TSP is rehydrated. They concluded that TSP did bind moisture during cooking.

However, researchers do not agree on how soy protein affects fat and moisture retention in ground beef during cooking. Seideman et al. (1977) found that ground beef patties containing (10, 20, or 30%) TSP retained more moisture but lost more fat during cooking than did all-beef patties; the findings of Anderson and Lind (1975), Drake et al. (1975) and McWatters (1977) were similar. All four of these studies used ground beef patties containing between 15 and 35% fat. However, Ziprin and Carlin (1976) found no difference in the moisture or fat content of cooked meatloaves between all-beef and 15% soy-substituted beef loaves. In several studies TSP in meatloaf bound fat (Williams and Zabik, 1975; Nielsen and Carlin, 1974; Shafer and Zabik, 1975). Carlin et al. (1978) found that retention of both fat and moisture increased when the level of TSP in meatloaf was increased from 15 to 30%. While the fat of the ground beef used in these meatloaves was in the same range as that of the beef patties discussed above, these meatloaves contained other ingredients such as reconstituted nonfat dry milk, egg, bread crumbs, and seasoning that may aid in the retention of fat and moisture. Also, the smaller size and shorter cooking time of the beef patties than the meatloaves may effect the nutrient losses.

For beef products extended with soy protein, data have been published for cooking yield and moisture and fat retention, but not for the retention of other nutrients. We, therefore, designed an experiment to (1) determine the nutrient composition of raw and cooked ground beef extended with three soy proteins, (2) determine the true and apparent retention of nutrients in cooked soy-extended beef, and (3) compare the true and apparent retention techniques for assessing the nutrients in cooked soy-extended meats.

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MATERIALS & METHODS

SEVEN FORMULATIONS of ground beef were mixed by a commercial meat packing company (Esskay, Baltimore, MD) and packaged in 4-oz patties (11.5 cm diameter; 1 cm thickness). One formulation was 100% ground beef. In each of the other six formulations, beef was extended with one of three soy proteins: flour (TVP, Archer Daniels Midland Company), concentrate (Procon 2060, A.E. Staley Manufacturing Company), or isolate (PP-220, Ralston Purina Company); with or without iron and zinc fortification. Only the soy flour was texturized and the size of the soy granules varied with flour being the largest and isolate the smallest. Two ground beef products were used in the beef patties. One contained 10% fat (Beef 90) and the other 50% fat (Beef 50). The six soy extended beef patties were 30% Beef 50 and 50% Beef 90, while the all beef patties were 31.5% Beef 50 and 68.5% Beef 90.

The analyzed protein contents of the three dry soy products were 53.5% (flour), 69.5% (concentrate) and 87.1% (isolate). During commercial formulation, each soy protein product was rehydrated to a protein level of 19% (N x 6.25). Each beef-soy formulation was 20% rehydrated soy and 80% beef. Grams of soy products required for 20 g of rehydrated flour, concentrate, and isolate were 7.10, 5.47, and 4.37, respectively. Each of the dry soy products (flour, concentrate and isolate) were fortified with iron (60 mg/100g, soy protein) as ferrous fumarate and zinc (25 mg/100g soy protein) as zinc oxide. These fortified soy proteins were rehydrated and commercially mixed with beef in the same proportions as the unfortified soy proteins.

Patties were frozen at the meat packing plant on the day of mixing and were stored at $-29°C$ ($-20°F$) until preparation for nutrient analysis. The patties had been stored for approximately seven months when the nutrient analysis were carried out.

Three raw patties of each of the seven ground beef products

underwent proximal analysis by Nutrition International Corporation (East Brunswick, NJ), and another three patties of each formulation were analyzed for mineral content (Ca, P, Mg, Na, Fe, Zn, Cu, Mn) by Hazleton Raltech, Inc. (Madison, WI). These determinations were carried out by standard AOAC methods (1980): moisture (7.003), protein (2.056–2.058), fat (14.140) and ash (7.009). Samples for mineral analysis were prepared by AOAC method 3.005 (AOAC, 1980) and minerals were determined by inductively coupled plasma-atomic emission spectrometry (Dahlquist and Knoll, 1978).

Six patties of each ground beef product were thawed in a refrigerator at 4°C (40°F) for about 20 hr and cooked on a broiler pan in a 170.7°C (350°F) oven for 20 min (10 min per side). Patties were cooked two at a time in a conventional gas oven (Garland Institutional) on a pan in the center of the oven. The oven temperature was always at 170.7°C (350°F) (oven thermometer) when patties were placed in the oven and when they were removed. Measurements of oven temperature during cooking were not made. Maximum internal temperature of the patties at the end of cooking ranged from 62–70°C (Mean ± SD: 65.3±1.86°C). Each patty was weighed before and after cooking, then submitted to the same nutrient analysis as were the raw patties. Three cooked patties of each formulation underwent proximal analysis and three different patties of each formulation were analyzed for mineral content.

True nutrient retentions (TR) were calculated as follows:

$$\% \text{ TR} = \frac{\text{Nutrient content/g of cooked food} \times \text{g of food after cooking}}{\text{Nutrient content/g of raw food} \times \text{g of food before cooking}} \times 100$$

Apparent nutrient retentions (AR) were calculated as follows:

$$\% \text{ AR} = \frac{\text{Nutrient content/g of cooked food (dry basis)}}{\text{Nutrient content/g of raw food (dry basis)}} \times 100$$

Appropriate data were subjected to statistical analysis of variance. When group means were found to be significantly different, Duncan's Multiple Range Test was used to determine which groups were significantly different from each other. True and apparent retentions were compared by Student's t test.

RESULTS & DISCUSSION

Yield

Percent yield after cooking differed significantly among the patty formulations (Table 1). There was an inverse relationship between cooking yield and refinement of soy protein used in the patties; flour was the least and isolate the most highly refined. Cooking yields of the beef extended with soy isolate (70.7 or 68.8%) were not significantly different from the all beef (69.3%). Yields of patties with soy ranked in the following ascending order, with or without fortification, respectively: soy isolate, 68.8 and 70.7%; soy concentrate, 74.8 and 77.1% and soy flour, 80.2 and 79.0%.

Ziprin et al. (1981) found that yield was significantly higher for beef patties extended with 10% soy (defatted flour, concentrate, or isolate) than for all-beef patties. McWalters (1977) found that cooking losses decreased and

Table 1—Percent yield after cooking all beef and beef extended with soy protein patties

	Patties[a]
All beef	69.3±1.8 DE
Beef with Soy isolate	70.7±1.2 D
Soy concentrate	77.1±1.3 B
Soy flour	79.0±1.3 A
Beef with Fe and Zn Fortification	
+ Soy isolate	68.8±1.1 E
+ Soy concentrate	74.8±1.4 C
+ Soy flour	80.2±0.8 A

[a] Mean of six patties ± S.D. Means with the same letter are not significantly different [Duncan's Multiple Range Test $p < 0.05$].

Table 2—Nutrient content of raw all beef patties and beef patties extended with soy protein[a]

		Beef + Soy isolate		Beef + Soy concentrate		Beef + Soy flour	
	All beef	w/o Added Fe and Zn	w/Added Fe and Zn	w/o Added Fe and Zn	w/Added Fe and Zn	w/o Added Fe and Zn	w/Added Fe and Zn
Protein (%)[b]	16.4 ±0.9	16.6 ±0.0	17.2 ±0.8	16.5 ±0.2	16.8 ±0.1	17.0 ±1.0	17.1 ±0.4
Fat (%)[b]	23.5 ±1.8	21.6 ±1.8	21.3 ±0.1	21.6 ±0.5	21.7 ±1.3	22.2 ±1.3	22.9 ±0.5
Moisture (%)[b]	58.7 ±1.3	60.5 ±1.8	58.9 ±0.5	60.0 ±0.5	60.3 ±0.5	58.5 ±0.2	58.6 ±0.4
Total ash (%)	0.7 ±0.1 E	0.7 ±0.0 DE	0.9 ±0.1 BC	0.9 ±0.0 C	0.8 ±0.0 CD	1.0 ±0.1 AB	1.0 ±0.2 A
Minerals (mg/100g)							
Ca	8.1 ±0.8 D	12.6 ±0.1 C	14.0 ±1.7 C	28.2 ±0.4 B	28.0 ±1.1 B	31.5 ±1.7 A	32.8 ±3.6 A
P	125.2 ±1.3 E	132.9 ±3.1 C	128.8 ±1.9 D	142.7 ±1.0 B	135.4 ±0.2 C	151.3 ±2.8 A	151.2 ±2.1 A
Mg	15.0 ±0.3 F	17.7 ±0.4 D	16.5 ±0.3 E	29.1 ±0.4 B	27.5 ±0.2 C	34.6 ±0.6 A	34.3 ±0.3 A
Na	65.8 ±1.9 C	102.6 ±0.4 B	109.0 ±4.9 A	53.2 ±0.2 E	57.6 ±0.8 D	53.0 ±0.3 E	54.0 ±0.9 E
Fe	1.8 ±0.1 F	2.2 ±0.8 E	4.0 ±0.0 B	2.0 ±0.0 E	3.8 ±0.0 C	2.3 ±0.1 D	4.2 ±0.2 A
Cu	0.045±0.003 D	0.091±0.004 C	0.103±0.016 C	0.129±0.004 B	0.121±0.006 B	0.175±0.009 A	0.182±0.121 A
Zn	3.8 ±0.1 C	3.2 ±0.1 D	4.0 ±0.0 B	3.0 ±0.0 E	3.9 ±0.0 B	3.2 ±0.1 D	4.2 ±0.0 A
Mn	0.01 ±0.0 G	0.07 ±0.0 F	0.08 ±0.0 E	0.28 ±0.006 C	0.26 ±0.006 D	0.29 ±0.006 B	0.30 ±0.0 A

[a] Mean of 3 samples ± S.D.
[b] No significant differences [ANOVA $p < 0.05$]. Means with the same letter (within a row) are not significantly different [Duncan's Multiple Range Test $p < 0.05$].

Table 3—Nutrient content of cooked all beef patties and beef patties extended with soy protein[a]

		Beef + Soy isolate		Beef + Soy concentrate		Beef + Soy flour	
	All beef	w/o Added Fe and Zn	w/Added Fe and Zn	w/o Added Fe and Zn	w/Added Fe and Zn	w/o Added Fe and Zn	w/Added Fe and Zn
Protein (%)[b]	21.5 ±0.9	22.7 ±1.3	21.9 ±0.6	21.0 ±0.6	21.9 ±1.5	21.6 ±0.8	20.6 ±0.1
Fat (%)[b]	23.4 ±1.0	21.1 ±0.5	22.0 ±2.8	20.6 ±1.4	20.1 ±1.0	21.1 ±0.6	21.0 ±0.6
Moisture (%)	52.5 ±0.6 D	54.2 ±0.6 BC	53.4 ±1.4 CD	55.2 ±0.9 AB	55.7 ±0.3 A	53.8 ±0.3 CD	54.3 ±0.3 BC
Total Ash (%)	0.8 ±0.1 D	0.7 ±0.2 D	0.9 ±0.0 CD	1.0 ±0.1 BCD	1.2 ±0.3 AB	1.1 ±0.1 ABC	1.4 ±0.2 A
Minerals (mg/100g)							
Ca	11.2 ±0.8 D	18.9 ±2.0 C	18.0 ±0.6 C	37.6 ±0.9 B	39.8 ±2.1 AB	40.0 ±2.1 AB	40.6 ±0.9 A
P	152.8 ±1.6 D	160.2 ±2.8 C	160.0 ±3.0 C	168.1 ±2.1 B	171.4 ±3.4 B	182.1 ±1.2 A	182.8 ±2.9 A
Mg	17.8 ±0.4 E	21.2 ±0.3 D	20.9 ±0.1 D	32.9 ±0.7 C	34.0 ±0.9 B	39.6 ±0.2 A	39.9 ±0.3 A
Na	66.2 ±1.7 B	105.5 ±2.4 A	107.9 ±5.9 A	67.1 ±3.7 B	61.6 ±2.8 BC	66.9 ±4.7 B	59.5 ±0.2 C
Fe	2.4 ±0.0 E	2.8 ±0.0 CD	5.6 ±0.3 A	2.6 ±0.1 DE	5.3 ±0.2 B	2.9 ±0.0 C	5.2 ±0.1 B
Cu	0.049±0.005 D	0.118±0.014 C	0.120±0.009 C	0.153±0.005 B	0.148±0.004 B	0.206±0.002 A	0.209±0.004 A
Zn	4.8 ±0.1 B	3.9 ±0.0 C	5.0 ±0.2 A	3.6 ±0.0 D	4.8 ±0.0 B	3.6 ±0.0 D	4.6 ±0.1 B
Mn	0.011±0.002 F	0.09 ±0.003 E	0.105±0.0 D	0.312±0.014 C	0.336±0.006 B	0.341±0.007 B	0.356±0.009 A

[a] Mean of 3 samples ± S.D.
[b] No significant differences [ANOVA $p < 0.05$]. Means with the same letter (within a row) are not significantly different [Duncan's Multiple Range Test $p < 0.05$].

cooking yield increased progressively as 5, 10, or 15% soy meal was added to ground beef patties (27% fat, 55% moisture) and baked in rotary oven at 177°C for 15 min. Bowers and Engler (1975) found that adding textured soy (15 or 30%) to ground beef (25% fat) also decreased cooking losses with the least loss at the 30% level. When Anderson and Lind (1975) added 25% hydrated textured vegetable protein to ground beef (15, 20, 25, and 35% fat), they found the cooking yields of the soy-extended beef slightly exceeded those of all beef patties.

Nutrient composition

The nutrient contents appear in Table 2 for raw ground beef patties and in Table 3 for cooked patties. Percent fat was similar in cooked and raw patties; however, moisture content decreased and protein content increased during cooking. There were no significant differences in protein, fat, or moisture among the seven raw patty types or in protein and fat among the seven cooked patties type. However, the seven patty formulations did differ significantly in mineral composition as shown in Tables 2 and 3. The mineral content of the patties decreased the more refined the soy protein added; isolate being the most refined, flour the least. These differences in mineral content of the patties can be attributed to the mineral content of the various soy proteins added.

Nutrient retention

There were no significant differences in protein, fat, total ash, Ca or Cu retention in any of the cooked all-beef patties or soy-extended beef patties (Table 4). True retention of moisture increased the less refined the soy added to the patties. Six of the eight minerals analyzed showed significant differences in true retention. In general, the more of a mineral present in the raw patties the higher was its true nutrient retention.

This study found no difference in the fat retention or fat content of cooked beef patties with or without soy protein. Ziprin et al. (1981) noted that published reports do not agree on the effect of soy protein ingredients on the fat content of cooked beef patties. They attributed the disagreement to differences in method of patty formulation, size and thickness of patties, cooking method, and the degree of doneness. Drake et al. (1975) found that the amount of fat lost when beef patties extended with rehydrated textured soy protein were grilled in an electric frypan depended on the original level of fat in the raw ground beef and not on the level of soy protein added. On the contrary, other investigators reported less fat retention in cooked patties containing soy protein than in all-beef patties (Anderson and Lind, 1975; Thomas et al., 1978).

Apparent retentions for all nutrients analyzed in cooked meat patties appear in Table 5. The % AR was higher than

Table 4—Percent true retention of nutrients in cooked all beef patties and beef patties extended with soy protein[a]

		Beef + Soy isolate		Beef + Soy concentrate		Beef + Soy flour	
	All beef	w/o Added Fe and Zn	w/Added Fe and Zn	w/o Added Fe and Zn	w/Added Fe and Zn	w/o Added Fe and Zn	w/Added Fe and Zn
Protein[b]	91.6± 2.5	96.7± 7.2	88.2± 3.1	98.5±3.5	98.5± 6.9	100.1±5.8	98.1±5.2
Fat[b]	70.3± 7.2	68.4± 7.1	71.5±10.2	74.0±6.8	70.3± 1.1	75.4±7.7	75.6±2.4
Moisture	62.7± 1.3 C	64.2± 1.4 C	62.8± 0.6 C	71.3±2.1 B	69.9± 2.3 B	72.5±1.5 AB	74.3±1.2 A
Total ash[b]	80.4±11.3	105.5±49.7	70.2± 5.7	87.5±4.5	112.8±26.3	92.6±4.3	99.7±9.6
Minerals							
Ca[b]	95.2±11.0	104.6±12.3	88.7± 8.4	102.3±0.6	105.3±10.1	100.7±5.5	99.6±9.1
P	83.8± 1.6 C	84.6± 5.1 C	85.0± 2.4 C	90.5±2.6 B	93.6± 2.0 AB	95.2±3.3 AB	96.5±2.0 A
Mg	81.8± 1.0 D	83.9± 5.3 CD	86.4± 1.4 BC	86.9±0.8 BC	91.6± 1.7 A	90.7±2.7 AB	92.8±0.9 A
Na	69.0± 1.9 E	72.1± 3.0 DE	67.9± 6.8 E	96.9±5.5 AB	79.1± 4.3 CD	100.1±8.8 A	88.0±2.0 BC
Fe	90.1± 4.7 C	90.4± 5.5 C	95.8± 3.0 BC	96.6±2.6 ABC	103.7± 2.9 A	97.3±2.1 ABC	99.3±5.8 AB
Cu[b]	75.7±12.1	91.1± 8.8	81.4±16.0	91.3±1.5	90.5± 7.3	93.5±6.2	91.6±4.5
Zn	88.4± 0.3 ABCD	85.0± 3.4 D	86.7± 2.2 CD	91.4±1.7 AB	92.0± 1.2 A	88.1±2.1 BCD	89.3±0.9 ABC
Mn	75.6±12.7 B	90.5± 4.6 A	89.8± 1.8 A	86.4±0.7 A	94.3± 0.8 A	94.0±2.5 A	94.8±1.5 A

[a] Values are means ± S.D. of three raw patties compared to three cooked patties.
[b] No significant differences [ANOVA $p < 0.05$]. Means with the same letter (within a row) are not significantly different [Duncan's Multiple Range Test $p < 0.05$].

Table 5—Percent apparent retention of nutrients in cooked all beef patties and beef patties extended with soy protein[a]

		Beef + Soy isolate		Beef + Soy concentrate		Beef + Soy flour	
	All beef	w/o Added Fe and Zn	w/Added Fe and Zn	w/o Added Fe and Zn	w/Added Fe and Zn	w/o Added Fe and Zn	w/Added Fe and Zn
Protein	113.9± 7.7[b]	118.1±12.0	112.6+ 7.9[b]	113.6±4.2[b]	116.6± 7.4[d]	113.9±6.2[d]	109.3± 5.3
Fat	87.0± 5.0[d]	83.3± 7.2	91.0±10.3	85.3±7.6	83.2± 1.7[c]	85.7±6.8	84.3± 2.4[d]
Total ash	100.5±18.5	94.5±10.7	89.6± 6.9[d]	100.9±5.6[d]	133.2±31.7	103.3±8.3	115.0±12.7
Minerals							
Ca	121.2±17.7	129.4±11.2	114.6±13.3[d]	119.2±2.1[c]	127.5±11.3	114.4±6.3[d]	112.1± 9.9
P	106.3± 3.3[c]	104.8± 5.9[d]	109.8± 5.1[b]	105.3±3.3[b]	113.5± 1.0[c]	108.1±2.1[b]	108.8± 3.0[b]
Mg	103.8± 4.0[c]	103.9± 6.7[d]	111.5± 4.0[c]	101.1±1.9[c]	111.0± 0.2[c]	102.9±1.4[b]	104.5± 1.9[c]
Na	87.6± 3.7[b]	89.3± 5.5[b]	87.5± 6.9[d]	112.7±5.0[d]	95.8± 7.1[d]	113.5±8.9	99.0± 1.4[b]
Fe	114.2± 3.5[b]	112.0± 5.6[b]	123.7± 6.2[b]	112.5±3.3[b]	125.6± 1.6[c]	110.5±2.4[b]	111.9± 6.0
Cu	97.0±13.9	111.9±13.9	103.8±21.8	106.2±0.2[c]	110.5±13.0	106.5±5.3	102.4± 4.6[d]
Zn	112.2± 4.8[b]	105.3± 3.8[b]	112.0± 4.9[b]	106.5±2.0[c]	111.4± 2.1[c]	99.9±2.6[b]	100.7± 1.3[c]
Mn	116.7±28.9	111.4+ 6.0[b]	113.6± 3.1[c]	100.9±1.6[c]	114.6± 2.7[c]	106.8±2.3[b]	106.9± 2.3[b]

[a] Values are means ± S.D. of three raw patties compared to three cooked patties.
[b] Significantly different from true retention ($p < 0.01$) (t-test).
[c] Significantly different from true retention ($p < 0.001$) (t-test).
[d] Significantly different from true retention ($p < 0.05$) (t-test).

the % TR of all nutrients examined in these meat products. This finding reaffirms the findings of Murphy et al. (1975) that apparent retentions tend to give falsely high values and do not allow for loss of solids in cooking. Therefore, weighing a food before and after cooking for the calculation of a true retention is the preferred method of determining the nutrient retention of a food product.

CONCLUSIONS

NO SIGNIFICANT DIFFERENCES in true retention of protein, fat, total ash, Ca or Cu were found between all-beef patties and beef patties extended with any of the soy proteins tested. True retention of moisture in the patties decreased as the refinement of the soy added to the patties increased. Six of the eight minerals analyzed showed significant differences in true retention. In general, the higher the mineral content of the raw patty, the higher its true nutrient retention. The mean ranges of TR for the beef patties were: protein, 88.2–100.1%; fat, 68.4–75.6%; moisture, 62.7–74.3%; ash, 70.2–112.8%; calcium, 88.7–105.3%, phosphorus, 83.8–96.5%; magnesium, 81.8–92.8%; sodium, 67.9–100.1; iron, 90.1–103.7%; copper, 75.7–93.5%; zinc, 85.0–92.0%; and manganese, 75.6–94.8%.

The % AR was higher than the % TR in all-beef patties and beef patties extended with soy protein for all the nutrients examined.

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Measurement of Oxidation-Related Changes in Proteins of Freeze-Dried Meats

Z. NAKHOST and M. KAREL

ABSTRACT

A new methodology for detection of lipid oxidation in freeze-dried meats, using myoglobin, has been developed. Fresh, cold beef was ground, freeze-dried and stored aerobically at 37°C. Samples, taken at different time intervals, were reconstituted and "meat extract" obtained. Extent of myoglobin insolubilization was determined by absorbance intensity at isobestic point (525 nm). Oxidation of oxymyoglobin to metmyoglobin in meat extract was quantified by measuring α peak intensity of metmyoglobin at 630 nm. Myoglobin polymerization was determined by isolation of myoglobin dimers and monomers from meat extract using gel filtration chromatography. Dimer/monomer ratio was calculated from Soret band absorption intensity at 409 - 415 nm. The three myoglobin-based oxidative indicators correlate well with each other and can be used to detect extent of lipid oxidation in freeze-dried meat products.

INTRODUCTION

OXIDATIVE DETERIORATION of freeze-dried meats is a major factor limiting the shelf life of such meat products. The reaction involves development of off-flavor compounds in lipids of meat. Also, extensive lipid-protein interactions cause changes in meat proteins which result in toughening of freeze-dried meat products (Tappel, 1956; El-Gharbawi and Dugan, 1965; Love and Pearson, 1971). The classical methods of measurement of the extent of oxidation such as determination of peroxide value and TBA value do not give adequate results in muscle foods (Chipault and Hawkins, 1971; Melton, 1983; Williams et al., 1983), and a need exists for improved methods.

In our earlier studies on the potential use of protein changes as indicators of oxidative deterioration we found that oxidation-induced changes in myoglobin in a model system containing metmyoglobin and methyl linoleate, could serve as the oxidation indicators (Nakhost and Karel, 1983). This paper reports the application of the previously developed myoglobin-based oxidative indicators for measurement of the extent of lipid oxidation in stored, freeze-dried meats.

MATERIALS & METHODS

Preparation of meat samples

Fresh, cold top round beef was processed as follows: 300g beef with 15 ± 2% fat content were cut into chunks and ground by pulsed rotating blade in a food processor for 45 sec. Ten gram samples of homogeneous comminuted meat were weighed into 50 mL glass containers, immediately frozen in liquid nitrogen, and dried in a Virtis freeze-drier. The dried samples were stored in closed containers at 37°C. Headspace of the storage containers provided large excess of oxygen (more than 4 moles of oxygen per mole of unsaturated fatty acids) to allow extensive oxidation of unsaturated fatty acids during storage. In order to study the progress of oxidative damage in myoglobin, the samples were tested after 0, 2, 6, 8, 10, 20 and 30 days of storage.

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Preparation of "meat extract"

After suitable incubation times, the freeze-dried meat samples were reconstituted to the original water content of 70%. For myoglobin extraction we added 1 mL deionized water per gram of reconstituted meat and vortexed for 1 min. The samples were stored for 30 min at 4°C, revortexed for 30 sec and centrifuged at 10,000 rpm for 30 min (2°C). The supernatant was filtered (using S&S filter paper #595) and recentrifuged at 10,000 rpm for 10 min (2°C). The clear supernatant, hereafter called "meat extract," was recovered from the centrifuge tube (using a Pasteur pipet). Fig. 1 shows the experimental flow diagram for preparation and analysis of meat extract.

Determination of myoglobin insolubilization

The absorption spectra of all meat extracts were scanned between 700 - 460 nm to obtain the characteristic peaks. The extent of myoglobin aggregation and insolubilization in the stored freeze-dried meat, was determined by obtaining the absorbance intensity at isobestic point (525 nm) of natural myoglobin derivatives.

Determination of myoglobin oxidation

Oxidation of oxymyoglobin to metmyoglobin in meat extract samples was quantified by measuring the α peak intenstiy of metmyoglobin (absorbance at 630 nm). To obtain this value, % myoglobin insolubilization (loss from the system) of each sample has been taken into account.

Isolation and determination of myoglobin polymers

Gel filtration chromatography was used to isolate myoglobin monomer and dimer from the rest of sarcoplasmic and extracellular (hemoglobin) proteins existing in the meat extract. The meat ex-

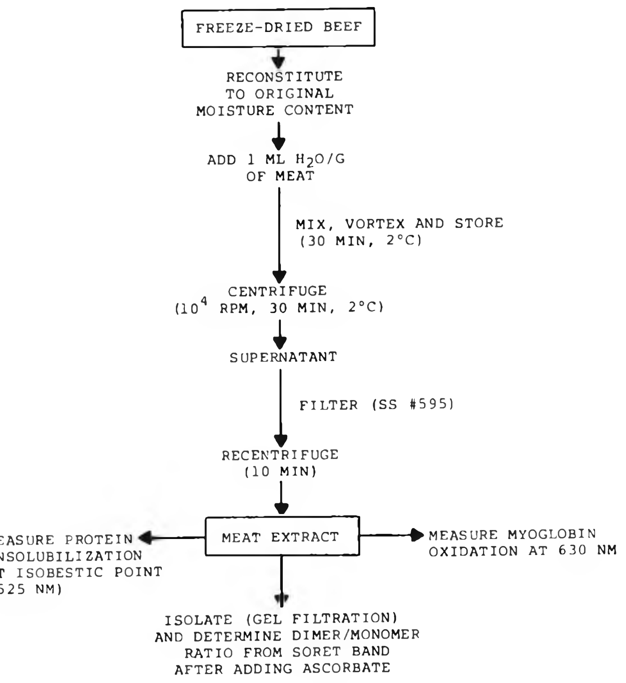

Fig. 1—Experimental flow diagram for preparation and analysis of meat extract.

tract (1 mL) of stored samples, after desirable dilution with buffer, was passed through a Sephadex G-100 superfine column (1 x 120 cm, fractionation range 4,000 - 100,000) and 1 mL effluent fractions were collected. Phosphate buffer, 0.05M, pH 7.45 was used for equilibration and elution of the column, the flow rate was 4 ml per hr. Molecular weight determiation of eluted proteins was achieved by column calibration using protein standards and the method of Catsimpoolas (1974).

After completion of fractionation, all monomer and dimer fractions were pooled separately. To eliminate turbidity after sample collection, the pooled fractions were passed through a low-protein binding (99% myoglobin recovery), microfilter (Millex-GV Filter units, SLGV025LS, with 0.22 μm pore size, Millipore Corp., Bedford, MA). The use of a microfilter for clarification of turbid meat extract has been reported by Gumpen and Fretheim (1983).

To determine the amount of myoglobin in the pooled, clarified monomer and dimer fractions, absorption spectra in the range of Soret band (460 - 360 nm) were obtained and the Soret band absorption intensity (409 - 415 nm) was measured. Since in the Soret band region the extinction coefficient of metmyoglobin is higher than that of oxymyoglobin, prior to Soret band determination, sodium ascorbate (550 ppm) was added to each fraction to convert back all the metmyoglobin to oxymyoglobin. The reduction reaction completed after about 30 – 45 min at room temperature.

RESULTS & DISCUSSION

Determination of myoglobin insolubilization

Since in meats myoglobin exists in a mixture of deoxy, oxy, and metmyoglobin, characteristic absorption spectra of none of them can be used to determine the total amount of myoglobin in meat extract. We found that by using absorption intensity at the isobestic point (525 nm), where the molar extinction coefficient of all above derivatives is equal (Fig. 2), we can directly determine the amount of myoglobin insolubilization in freeze-dried meat.

There was a pronounced increase (18%) in myoglobin insolubilization after the first 2 days of storage. This value reached 38% after 10 days and 62% after 30 days of storage (Fig. 3 and 6). The pattern of myoglobin insolubilization in this meat system correlated well with myoglobin insolubilization in our model system (Nakhost and Karel, 1983).

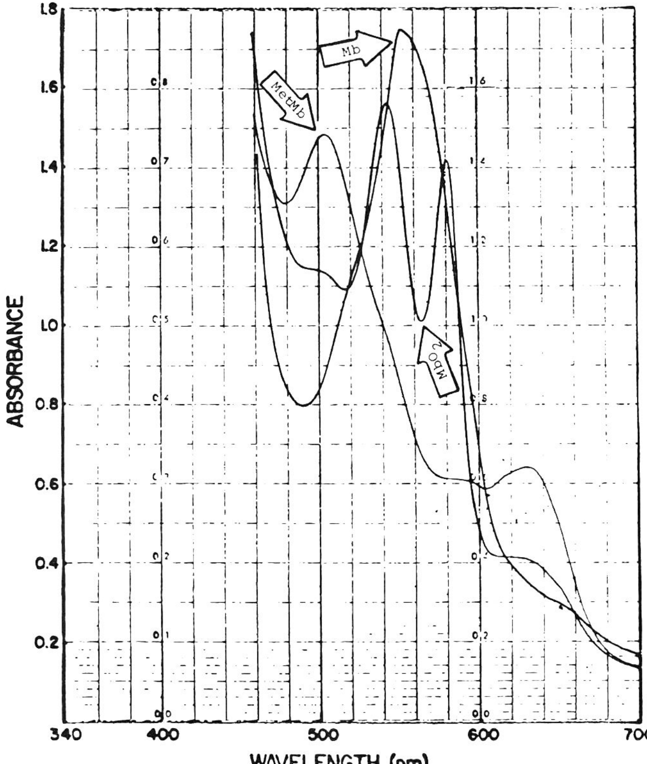

*Fig. 2—Absorption spectra (range of 0 - 2.0) of: *Fresh meat extract (MbO_2); *+ potassium ferricyanide (MetMb); *+ sodium hydrosulfite (Mb). Spectra of all the above cross at the isobestic point (525 Mb).*

Determination of myoglobin dimer/monomer ratio

Myoglobin dimer to monomer ratio can be used as an "internal standard" for detection of lipid oxidation in freeze-dried meats. Comparison of the Soret band intensity (409 - 415 nm) of dimer fraction isolated from *unstored* freeze-dried meat, with those obtained from *stored* freeze-dried meat samples showed a progressive increase in the amount of dimer fraction upon storage (Fig. 4). On the other hand, the comparison of the Soret band intensity (409 - 415 nm) of monomer fractions showed a gradual decrease, with storage time (Fig. 5). Our calculations showed the ratio of myoglobin dimer to monomer to be 1% just after freeze-drying [Van Den Oord et al. (1969) reported values in the range 0.7 - 1.8% depending on different extraction procedures and treatments] and to increase to about 8% after 10 days and 8.6% after 30 days of storage (Fig. 6).

The above results prove the utility of a novel methodology for detection of lipid oxidation in freeze-dried meats based on indirect effect of lipid oxidation on a representative protein. Three parallel oxidative indicators, namely: (1) progressive myoglobin insolubilization, (2) gradual oxidation of oxymyoglobin to metmyoglobin, and (3) increaseing ratio of dimer to monomer, were found to detect and measure the extent of lipid oxidation in freeze-dried meats. Measurement of myoglobin insolubilization using isobestic point method (525 nm) has the potential for determination of protein insolubilization due to any damaging factor, during meat processing and storage. The α peak intensity of myoglobin (A_{630nm}) allows to follow the progress of myoglobin oxidation as a result of lipid oxidation. Soret band measurement provides a very sensitive and reliable method for detection of very small amounts of myoglobin dimer. The three oxidative indicators can be obtained from only one sample and show good correlation with each other.

To optimize the isolation method, we are currently investigating the application of HPLC in separation of myoglobin monomer and dimer from meat extract. We believe this methodology to have extensive applications in objective determination of lipid oxidation in freeze-dried meat products both in research and industry.

Determination of myoglobin oxidation

Oxidation of oxymyoglobin to metmyoglobin can occur as a result of lipid oxidation (Lin and Hultin, 1977). Comparison of the spectrum obtained from unstored freeze-dried meat with the spectra of the stored meat samples showed a progressive oxidation of oxy to metmyoglobin, upon storage (Fig. 3). Myoglobin in meat extract obtained from freeze-dried meat was almost entirely in the form of oxymyoglobin with absorbance value at 630 nm (α peak of metmyoglobin) to be 0.19. On the other hand myoglobin obtained from meat samples stored for 10 days, was quite oxidized and had the absorbance value of 0.66 (calculated based on 100% myoglobin recovery in meat extract). The absorbance value of meat samples reached 0.8 after 30 days of storage (Fig. 6).

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Fig. 3—Absorption spectra (range of 0 - 2.0) of extract obtained from reconstituted freeze-dried meat, after 2, 6, 8 and 10 days of storage. Spectrum with maximum intensity corresponds to unstored freeze-dried meat. Spectrum with minimum intensity corresponds to freeze-dried meat after 10 days of storage.

Fig. 4—Soret band spectra (range of 0 - 0.1) of dimer fraction isolated from stored freeze-dried meat, after 2, 6, 8, and 10 days of storage. Spectrum with minimum intensity corresponds to unstored freeze-dried meat. Spectrum with maximum intensity corresponds to freeze-dried meat after 10 days of storage.

Fig. 5—Soret band spectra (range of 0 - 1.0) of monomer fraction isolated from stored freeze-dried meat, after 2, 6, 8, and 10 days of storage. Spectrum with maximum intensity corresponds to unstored freeze-dried meat. Spectrum with minimum intensity corresponds to freeze-dried meat after 10 days of storage.

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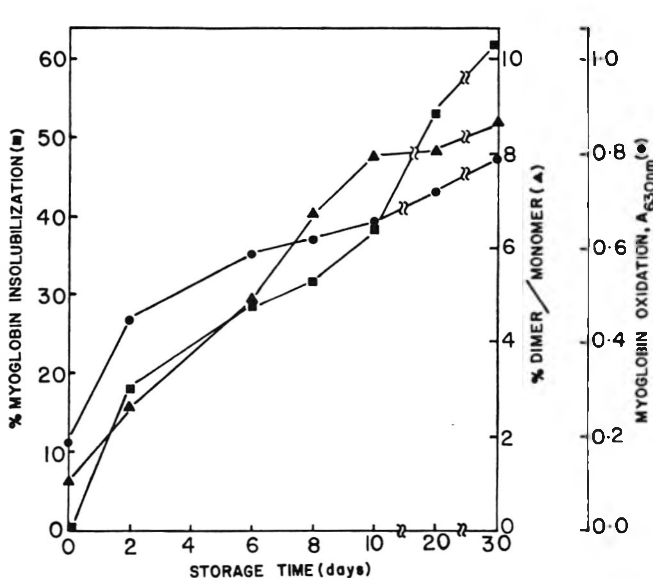

Fig. 6—Changes induced by lipid oxidation on myoglobin extracted from freeze-dried beef, upon storage.

Aging of Frozen Parts of Beef

TAMAR COHEN

ABSTRACT

Due to religious regulations, the beef imported into Israel is frozen soon after boning. In a search for a method for optimal aging of the beef after thawing and koshering, 8 different aging experiments were conducted, from 0-12 days. Cooking loss and myofibril fragmentation index were found to be more affected by the freezing preceding aging. Nonprotein nitrogen components were found to increase during optimum aging and then decrease. Sensory evaluation of tenderness, performed by multiple-paired comparisons, indicated a significant improvement in meat tenderness after 1 day of aging and further gradual improvement up to 7 days.

INTRODUCTION

THE TENDERNESS OF BEEF is an important palatability factor for consumer acceptance. There are a number of factors that influence meat tenderness (Szczesniak and Torgeson, 1965); one of the important ones is aging. Usually aging occurs before freezing the meat. Marsh et al. (1968) found that freezing lamb meat during rigor, before completion of aging leads to toughness. Locker et al. (1975) concluded that quick-freezing of meat before aging is responsible for pronounced toughness and, therefore, they recommended conditioning and aging the meat before freezing.

In Israel, most of the beef consumed is imported frozen as primary cuts from the fore-quarters. Because of religious regulation, the young bulls are kosher-slaughtered and the meat is frozen soon after slaughter. Usually the carcasses are chilled for a maximum of 24 hr, deboned and quick-frozen. The cuts are kept frozen until consumption either domestically or in institutions (hospitals, army, kibbutzim, etc). Usually the meat is thawed, koshered and cooked as soon as possible. Many customers complain that the cooked meat is tough even though it may be cooked for a long time. We assumed that the reasons for this phenomenon is the freezing of the beef during or before rigor. Most of the studies reported in the literature on aging beef were conducted on the meat before freezing, either on the whole carcass or on deboned cuts. No studies exist concerning our problem. This paper deals with yet unexplored problems of aging quick-frozen kosher meat. This investigation was undertaken in order to determine a method for overcoming this problem by aging the beef after thawing and koshering and to follow the changes induced in the meat during this aging.

MATERIALS & METHODS

Meat

The frozen meat was bought in the original package as sold to the customers. The details of the experiments are outlined in Table 1. The origin of the meat for experiments 1-6 was Australia and was from the same source (Tancred); the meat for experiments 7-8 was Argentina. Experiment 8 was conducted on 4 cuts of shoulder in order to compare various sources of the same cut.

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Preparation for cooking

The frozen meat was cut with an electric saw into two to five blocks. Each block was cut into five pieces of 150–200g each and pieces were chosen randomly for treatments, wrapped with polyethylene film, and returned to the freezer. The pieces were thawed in a cold flow of air (the time needed was dependent on the size of the piece, generally about 2 hr). "Koshering" is a process conducted on thawed meat to remove the blood. The meat was soaked in cold tap water for 1 hr, salted generously with coarse salt (no specific percent), placed in a colander for 1 hr to allow the blood to drip out and lastly, rinsed well. Aging was begun immediately following the koshering process. The koshered pieces were wrapped with polyethylene film and refrigerated for aging at 5-6°C for the pre-determined number of days as outlined in Table 1. The drip loss at thawing was about 5%, but the weight loss was regained during koshering.

Cooking the meat

After the meat was aged for the required time, all the pieces were cooked simultaneously. The treatment without aging (0 days) was cooked immediately following koshering. The cooking method was adapted to the particular cuts: most of the cuts were cooked in moist heat (braising, boiling, stewing) and the choice ribs (exp. 2) were cooked in dry heat (pan-broiling for 2 min on each side), the cooking was terminated when the pieces were tender. Each piece was weighed before and after cooking. The cooked pieces were cubed with no apparent connective-tissue and kept warm until tasted.

Sensory evaluation

Using the "multiple-paried comparison" method (Larmond, 1977; Larmond et al., 1969), the same 8-10 untrained panelists participated in each session. Each received 10 coded pairs of all the combinations of five treatments in random order. Each sample consisted of two meat cubes of approximately 10g each and the panelist was asked to designate the more tender sample in each pair. The meaning of tender vs tough was discussed prior to the beginning of the experiment. The analysis was done according to the statistical models of Bradley and Terry (1952), and developed into a computer program by "Agriculture Canada" (Dietrich, 1968). (The program

Table 1—Details of the experiments for aging frozen beef

No. of exp.	Retail parts	Main muscle	Cooking method	Treatment (Days)	No. of repl.
1	fore ribs (chuck)	serratus ventralis	braising	0 – 12	5
2	choice ribs (entrecote)	longissimus dorsi	pan broiling	0 – 12	5
3	shoulder	triceps brachii	braising	0 – 9	4
4	flat ribs	latissimus dorsi	boiling	0 – 9	4
5	shoulder	infra spinam	Swiss steak	0 – 9	5
6	shin	extensor carpi radialis	stew	0 – 7	4
7	shoulder	triceps brachii	braising	0 – 7	2
8	shoulder	infra spinam	braising	0 – 7	8

was kindly supplied by the Department of Agriculture, Canada). The data were computed, analyzed and the maximum liklihood estimates of the preference parameters were obtained. The estimates were converted to logarithms in order to permit overall comparison on a linear scale (Bradley and Terry, 1952) and to assess significance of the estimates. The higher the estimates of the preferences, the better the treatment.

Myofibril fragmentation index

Myofibril Fragmentation Index (MFI) was determined as outlined by Olson et al. (1976). Samples of meat were taken for this test before cooking.

Nonprotein nitrogen

Nonprotein nitrogen components were determined in trichloroacetic acid (TCA) extracts as outlined by Strange and Benedict (1978). Tyrosine value was determined by the method of Pearson (1968). Creatinine levels were determined as outlined by Strange and Benedict (1978). Samples of meat were taken on various days of aging and immediately blended for the TCA extract.

Microbiological assays

Bacterial counts were performed according to Israel Standard Organization test methods (Anon., 1977) on samples of the meat at various stages: upon thawing, after koshering and after predetermined days of aging. A square of 2 x 2 cm, apprixmately 2 mm thick, was asceptically cut and shaken for 15 min in 40 mL sterile 0.1% peptone. Appropriate dilutions were made and samples were mixed with any one of the following media in Petri dishes and incubated as indicated. The counts were done on three media: (1) Nutrient agar 30°C for 3 days, (2) Violet red bile agar, 35°C for 1 day, (3) Rogosa agar, 24°C for 5 days; all dilutions were plated in triplicate and counted. The means of each triplicate was calculated. No statistical differences were found between means (Anon., 1977).

RESULTS & DISCUSSION

Cooking the meat

Eight experiments were conducted in studying the optimum duration of aging of thawed koshered meat; the details are outlined in Table 1. Each experiment consisted of five treatments, the first always without aging (0 days). In experiments 1 and 2, the aging was up to 12 days. As the meat had some off-odor on day 12, the duration of later experiments was shortened to 9 days. On 9 days of aging the uncooked meat had some off-odor, though the cooked meat had no off-flavor. After establishing that the meat was already tender in less than 7 days, it was decided to study in detail the changes during the first 7 days of aging (see exp. 6–8).

Cooking loss

In all experiments, cooking loss was calculated as percentage of the uncooked weight. The results are sumamrized in Table 2. In most experiments there was no difference between the treatments. Only in experiment number 5 was a significantly higher loss shown on day 0 of aging. In all the experiments in which the *meat was* cooked in moist heat, the cooking loss was quite high, reaching the range 32-40%, whereas in the dry heat experiment (no. 2), the cooking loss was less, average 18.8%.

The cooking loss is a result of denaturation of the proteins and is influenced by the temperature, time of cooking and water-holding capacity (WHC) of the meat (Locker and Daines, 1974). Postmortem glycolysis lowers the WHC (Lawrie, 1968), and aging improves it (Parrish et al., 1969). Therefore, we initially assumed that meat after aging will have less cooking loss. However, our findings indicate that changes induced by freezing probably have more influence on cooking loss than the extent of aging.

Myofibril fragmentation index

In experiments 3–8, the MFI was determined. The results are summarized in Table 3. In most experiments no significant differences were found in MFI between the treatments; only in experiments 3 and 7 was a significant rise in MFI on long aging shown. All the studies for assessment of MFI as an indicator of meat tenderness, (e.g., Moller et al., 1973; Olson and Parrish, 1977; Olson et al., 1976) were performed on unfrozen muscles. In most studies, a correlation was shown between the MFI and the tenderness of the meat in sensory evaluation and in shear force values. The aged meat was always found to have higher MFI values. As this study was conducted on frozen-thawed and aged muscles, it is probable that the long storage in a frozen state and subsequent thawing promoted the fragmentation during the blending of the muscle, even in the unaged meat, providing, as well, the reason for high MFI values in some of the experiments in all of the treatments. Therefore, we conclude that MFI is not an appropriate measure of predicting tenderness in meat frozen before measurement of its fragmentation index.

Nonprotein nitrogen (NPN) components

The NPN extract was tested for tyrosine and creatinine values on three parts of beef shoulder: triceps brachii, infra spinam, and supra spinam. The results are summarized in Fig. 1. At first there was a slight decrease in the tyrosine values (10%) during the koshering; however, tyrosine values increased at different rates up until days 4–7 of aging and declined after more than 7 days (this period of aging was tested in only one sample). The creatinine value increased significantly in one of the parts and in the other part declined after more than 7 days of aging. Pearson (1968) found an increasing tyrosine value in meat stored at 5°C up to 9 days. Khan and Van den Berg (1964), who studied

Table 2—% of cooking loss in the beef after aging^{a,b}

Treatment Experiment^{c}	Days of Aging 0	1	2	5	7	9	12	Mean
1	39.6 ± 2.6		34.3 ± 1.0	32.7 ± 1.8		32.2 ± 0.8	33.2 ± 2.0	34.1 ± 1.1
2	25.8 ± 3.0		13.9 ± 3.9	18.5 ± 5.8		18.4 ± 3.8	17.7 ± 1.7	18.8 ± 1.9
3	38.3 ± 0.9		36.8 ± 2.4	37.0 ± 0.7	40.0 ± 2.0	37.3 ± 0.8		37.8 ± 0.7
4	32.9 ± 2.4		30.4 ± 3.2	34.0 ± 3.0	35.6 ± 1.2	31.2 ± 3.5		32.8 ± 1.1
5	37.9 ± 1.6^{a}		34.6 ± 0.5^{ab}	33.1 ± 0.8^{b}	34.9 ± 1.1^{ab}	34.1 ± 1.0^{ab}		34.9 ± 0.5
6	32.1 ± 1.7	33.8 ± 2.2	36.9 ± 1.6	37.3 ± 6.5	34.3 ± 2.3			34.9 ± 1.1
7	38.9 ± 0.9	39.6 ± 3.0	40.2 ± 1.8	38.4 ± 1.3	40.1 ± 1.1			39.4 ± 0.5
8	39.7 ± 0.7	39.4 ± 1.3	37.7 ± 1.3	38.2 ± 0.9	37.7 ± 0.6			38.5 ± 0.4

[a] Results are presented as average ± SEM.
[b] Different letters in the line indicate significant differences, $p < 0.05$
[c] Details on the experiments are given in Table 1.

Table 3—Myofibril fragmentation index of the beef after aging[a,b]

Treatment Experiment[c]	Days of aging 0	1	2	5	7	9	mean
3	48.3 ± 5.2[a]		59.7 ± 3.4[ab]	81.3 ± 5.4[c]	64.0 ± 2.3[ab]	70.8 ± 3.8[bc]	64.8 ± 3.1
4	21.3 ± 6.5		21.5 ± 6.0	30.3 ± 2.1	22.8 ± 2.9	32.3 ± 1.8	25.5 ± 1.9
5	22.0 ± 8.5		45.8 ± 4.0	33.2 ± 18.7	53.9 ± 7.8	40.1 ± 4.9	39.6 ± 4.2
6	58.3 ± 10.8	59.7 ± 1.3	46.8 ± 3.9	62.8 ± 8.5	50.3 ± 5.3		55.2 ± 3.1
7	28.7 ± 3.5[a]	19.1 ± 1.4[a]	29.1 ± 1.4[a]	26.5 ± 3.5[a]	40.5 ± 2.1[b]		28.7 ± 2.5
8	61.0 ± 6.8	64.6 ± 9.6	67.6 ± 3.5	67.1 ± 3.2	70.7 ± 4.7		66.1 ± 2.4

[a] Results are presented as average ± SEM
[b] Different letters in the lines indicate significant differences, $p < 0.05$
[c] Details on the experiments are given in Table 1.

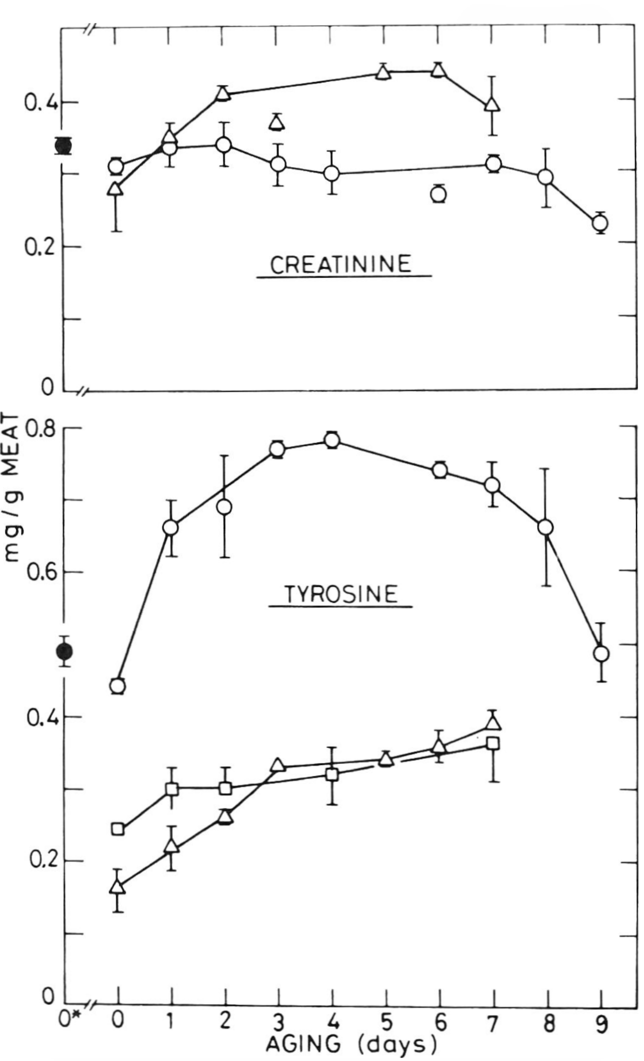

*Fig. 1—Creatinine and tyrosine content in the meat during aging, mean values ± standard deviations. * before koshering (supra spinam); △ shoulder (triceps brachii); □ shoulder (infra spinam); ○ shoulder supra spinam); ● shoulder (supra spinam) before koshering.*

meat in aseptic storage, concluded that tyrosine value is a good measure of proteolysis in the meat and not just a means of estimating the microbial activity as suggested by Strange and Benedict (1978) and Strange et al. (1977). In this study, the peak in tyrosine value coincides with the attaining of maximum tenderness in the muscle. Creatinine is a metabolite of creatine postmortem and is one of the flavor-enhancing components in meat. This reaction proceeds during cooking and was found to be an index to good sensory quality of meat (Macy et al., 1970). We found the

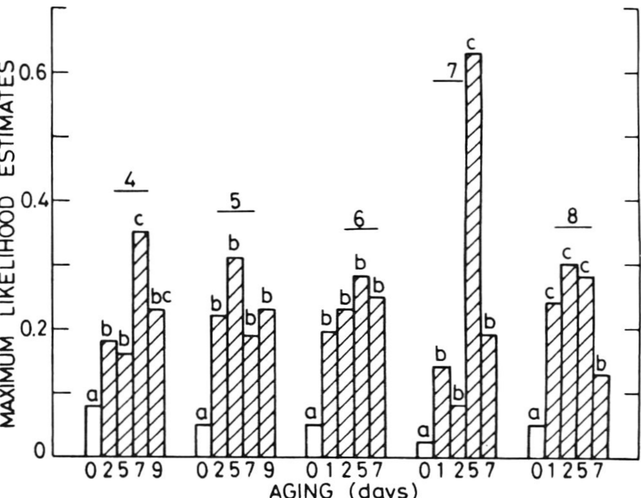

Fig. 2—Maximum liklihood estimates of preference parameters. Sensory evaluation of meat tenderness was conducted by the multiple paired comparison method in Exp. 4-8. Different letters indicate significant differences $p < 0.05$.

decreasing tyrosine and creatinine levels to coincide with the off-odor of the uncooked meat thus suggesting that both resulted from undesired decomposition processes.

Sensory evaluation of the meat texture

Initially, in experiments 1-3, the panel scored the texture on a category scale of 1 to 7 (1 = very tough, 7 = very tender). The results were too variable and no conclusions were reached. We believe the reasons were variability in the connective tissue components and the inexperience of the panel. As we were interested in assessing changes in the texture due to aging and not tenderness per se, we found that paired-preference tests were more suitable for determining difference in tenderness. We conducted multiple paired comparison tests with the results summarized in Fig. 2. In all experiments, treatment no. 1, without aging, was significantly tougher than all other treatments. In all of the experiments, there is a gradual improvement in tenderness up to 5 or 7 days. Significant improvements in tenderness were found in exp. 4 in 7 days and in exp. 7 in 5 days.

In two experiments (no. 7 and 8), tenderness decreased after 7 days of aging. The meat was so tender that the cooking time required for the other treatments was too long.

The normal aging, before freezing, is usually performed at 1-2°C. Smith et al. (1978) aged beef carcasses at 1 ± 1°C for 5-28 days and found that optimum quality was attained after 11 days with no pronounced improvement on longer aging. In New Zealand (Locker et al., 1975), the preference is for conditioning and aging at 10°C for 4 days; condition-

ing 1 day before boning, 3 days aging the cuts, and then freezing.

Very few studies exist on aging meat after freezing and thawing. Locker and Daines (1973) studied beef neck muscle that was conditioned 1 day at 15°C and then frozen. Subsequent aging at 15°C for 1 day was sufficient for tender meat. Winger and Fennema (1976) found that freezing for 4 hr at −3°C prior to normal aging (2 or 3 days at 15°C) accelerated the aging process.

Microbiological assays

In the assays, frozen beef had a small number of bacteria (less than $10^2/cm^2$). After koshering, the number of bacteria was somewhat higher. Up to 4 days of aging there was not a noticeable growth of bacteria; from 5 days on, we found total aerobic mesophiles – 10^6 to $10^8/cm^2$; coliforms up to $10^5/cm^2$ and lactobacilli up to $10^6/cm^2$. The counts up to 4 days indicate that the meat was not spoiled and even later the counts are not indicative of spoilage, but of an off-odor. Other studies (Elliot and Straka, 1964) showed that the rate of deterioration in refrigerated meat was the same in fresh meat and in frozen-thawed meat.

CONCLUSIONS

FROZEN BEEF imported into Israel as unaged parts is tough if cooked immediately upon thawing; cooking procedures do not overcome this toughness even with longer cooking times. We recommend aging the thawed and koshered meat in a refrigerator or cold room (5-6°C) wrapped in polyethylene film for at least 1 day but preferably for 4 days in order to ensure tender meat.

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Effect of Consuming Yogurts Prepared with Three Culture Strains on Human Serum Lipoproteins

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ABSTRACT

Ten human adult males' usual diets were modified by incorporating 681g nonfat, unpasteurized yogurt daily throughout three 14–21 day periods. A different set of select culture strains, two commercial and one patented, were used for yogurt production in the three dietary periods. Including yogurt daily in the diet significantly reduced fasting total serum cholesterol 10–12% in human adult males on some days, but serum cholesterol returned towards control values with continued yogurt consumption. Serum triglycerides and the proportions of serum lipoproteins were not significantly influenced by increasing yogurt consumption. Differences in concentrations of uric, orotic and hydroxymethylglutaric-like acids in the yogurts were insufficient to account for the differences in temporary hypocholesterolemic effects of yogurt consumption seen between strains.

INTRODUCTION

YOGURT is widely regarded as a nutritious and/or therapeutic food. Early in the 20th century Metchnikoff (1907) suggested that yogurt bacteria would combat atherosclerosis and a range of other illnesses. Examination of the literature shows that both milk and yogurt consumption have an inconsistent effect on serum cholesterol (Gold and Samuel, 1965; Mann and Spoerry, 1974; Mann, 1977a; Hepner et al., 1979; Rossouw et al., 1981; Massey, 1984). Sources of variation may be differences in levels of hypocholesterolemic compounds in milk and/or yogurt and different bacterial strains used in yogurt fermentation. Recent evidence suggests that three organic acids are likely hypocholesterolemic agents. Hydroxymethylglutaric (HMG) and orotic acids lower serum cholesterol when fed to rats (Beg and Siddiqi, 1968; Bernstein et al., 1977) and humans (Robinson and Dombrowski, 1983), while uric acid has been identified as a cholesterol synthesis inhibitor from human milk (Ward et al., 1982).

Commercial yogurt production generally utilizes a 1:1 mixed starter culture of only *Streptococcus thermophilus* and *Lactobacillus bulgaricus*. Numerous strains of each species exist and each strain exhibits different flavor, fermentation and coagulation properties (Deeth and Tamine, 1981). If a hypocholesterolemic "milk factor" (MF) does exist, this factor may be produced by only certain bacteria and all culture strains used in commercially produced yogurts may not utilize bacteria that produce or contain MF. This idea is supported by Mann (1979) who reported *Pseudomonas fluorescens*, normally uncommon in commercially produced yogurt, is capable of producing MF and by the observation that fermentation of milk by some strains of *S. thermophilus* and *L. acidophilus* produces hypocholesterolemic effects when the fermented product is consumed by mice and rats (Rao et al., 1981; Gruenewald, 1982; Pulusani and Rao, 1983).

This study was designed to test the hypothesis that different yogurt cultures may influence serum lipid concentrations differently, and these differences may be due to the variable concentrations of three potentially hypocholesterolemic acids, hydroxymethylglutaric, orotic and uric, in the yogurt.

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MATERIALS & METHODS

Experimental design

A dietary intervention study was conducted in three experimental periods. Each period corresponded to a set of select culture strains used in yogurt production. The subjects were asked to consume 681 ± 2g nonfat, unpasteurized yogurt daily. The yogurt had a total solids content equivalent to 0.8L skim milk. The first and second periods, CH-I and CH-II, lasted 14 days and the final period, SH-III, lasted 21 days. Twenty-one days were allowed between dietary periods to reestablish usual serum cholesterol values, since Mann (1977a) had reported that the hypocholesterolemic effect of yogurt persisted for several days after usual diets were resumed.

The subjects' weights were recorded prior to each blood collection. A constant body weight and level of exercise were encouraged throughout all study periods. The subjects were given subjective questionnaires weekly to report changes in exercise, diet, stress, health, and tolerance to yogurt.

A crossover design where different yogurts would be consumed in the same period by different subjects was not feasible in this study, as yogurt has a shelf life of about three weeks under refrigeration (Tamime and Deeth, 1980).

Subjects

Ten adult males, ages 23–39 years, participated in the study. All subjects were volunteers associated with the College of Home Economics. All lived at home during the entire study duration. All subjects were healthy with no history of coronary heart disease, high blood pressure, diabetes, or hyperlipidemia. Two of the subjects were lacto-ovo vegetarians and one subject smoked cigarettes. All ten subjects were within the desirable range of weight for height published by the Metropolitan Life Insurance Company (1983). All subjects had an initial serum cholesterol value within the normal range of 140–260 mg/dL, the 5th to 95th percentile for males of these ages according to data from the United States National Health Survey (Abraham et al., 1978). Only eight subjects participated in the first dietary period. Two additional applicants were recruited who participated only in the second and third dietary period.

Informed consent was obtained from all participants according to Washington State University policies regarding the use of human subjects in research.

Yogurt consumption

The subjects were allowed to consume the yogurt ad lib throughout the day or as a meal replacement as each desired. As the yogurt was unflavored subjects were permitted to add flavoring and sweeteners ad libitum. The amounts of added sweeteners were recorded by the subjects in their dietary records. Subjects were informed of the importance of consuming the same ratio of carbohydrate, protein, and fat throughout the study. Prior to yogurt consumption periods, each subjects' usual diet was recorded and analyzed to determine how each subject could incorporate yogurt into his diet as a replacement food in order to keep proportions of dietary fat, protein and carbohydrate constant.

Dietary records

The subjects recorded their daily intake for one usual diet and three yogurt periods. Each period included four days: Sunday through Wednesday. Hanson Dietetic scales (model #1460) were provided for each subject. Subjects were given written and verbal instruction on how to keep accurate dietary records.

The dietary data were computer analyzed using the Basic Data Set, Department of Agriculture Handbook No. 8, Dietary Information Tape for line items 1-4546. The data had been supplemented by Washington State University (WSU) for oils, some convenience foods, dairy products, and vitamin/mineral supplements.

Yogurt culture strains

Three strains of each of the *L. bulgaricus* and *S. thermophilus* cultures were used to produce four batches of yogurt. Two independent sets of selected commercial strains (CH-I and CH-II) were obtained from CHR Hansen's Laboratory, Inc., Milwaukee, Wisconsin. Strains CH-I and CH-II were freezed-dried samples of 1:1 ratio of *L. bulgaricus* and *S. thermophilus*. The third group was selected patented strains (SH-III) with a 1:1 ratio of *L. bulgaricus* 202 and *S. thermophilus* EBC and were supplied by Dr. K.M. Shahani, Univ. of Nebraska, Lincoln, NB.

Culture strain SH-III was used to produce two batches of yogurt, which are identified as SH-IIIA and SH-IIIB. During the final experimental period SH-IIIA was consumed through day 14 after which SH-IIIB was consumed from day 15 until day 21.

Yogurt production

The yogurt was made in four batches, two to three days prior to day 0 of the corresponding experimental period. A 14-day yogurt supply (114 kg) was prepared in the first three batches, while batch four was a 7-day supply. All subjects consumed yogurt made from the same set of selected strains within the same dietary period.

The yogurt was prepared at the Washington State Unviersity Creamery. Total milk solids content was increased to 13.5% by adding approximately 4.8 kg (4.5%) commercial nonfat milk powder to 107 kg liquid skim milk. At 49°C the mixture was homogenized at 1400 psi, and heated to 85°C for 30 min. The mixture was cooled to 43°C. Each inoculum was prepared with one set of selected culture strains (CH-I, CH-II, or SH-III). The bulk starter culture was incubated until reaching a predetermined activity level (ability to produce a pH 4.6–4.8 in milk within 2–4 hr). In all batches a 2% inoculum was added. The inoculum was carefully mixed with the fortified skim milk to assure uniform inoculation. The inoculated warm yogurt mix was poured into containers and incubated at 41–43°C until reaching a titratable acidity of 0.85% and pH 4.5. Incubation time ranged from 3.5–5.25 hr. After incubation, the yogurt was cooled by placing at 4°C. Final titratable acidity and pH were assayed between 18 and 24 hr post-refrigeration (Table 1). The final protein, fat, and carbohydrate content of each yogurt is also listed in Table 1.

Blood collection

Fasting blood samples were drawn on days 0, 7, and 14 of each experimental period and also on day 21 of period SH-III. One serum aliquot was immediatley stored at − 10°C for later triglyceride analysis. Unfrozen sera were stored at 4°C and analyzed for total cholesterol and high density lipoprotein (HDL) cholestrol within 48 hr and 24 hr, respectively. Sera were electrophoresed using the method of Hatch et al. (1963) within 3 hr of collection, except when equipment failure required that aliquots of sera collected on day 7 and 14 of period SH-III be fresh frozen and electrophoresed within 2 wk.

Lipid determination

Total serum cholesterol was measured enzymatically using a liquid reagent and high density lipoprotein (HDL) cholesterol was quantitatively determined using a kit (Beckman Instruments, Inc., Brea, CA). Dextran sulfate and magnesium acetate precombined in tablet form precipitated the low-density lipoproteins (LDL), very low density lipoproteins (VLDL), and intermediate density lipoproteins (IDL) fractions. After centrifugation, the supernatant containing HDL cholesterol was analyzed for cholesterol. Serum triglyceride levels were determined by the procedure used by Gottfried and Rosenberg (1973). Serum low density lipoprotein cholesterol (LDL-C) was calculated following the equations of Friedewald et al. (1972).

Organic acids

The concentration of hydroxymethylglutaric, orotic and uric acids in the study yogurts were determined as in Haggerty et al. (1984).

Statistics

Two-way analysis of variance was used to determine variation within and between dietary periods for body weight, total serum cholesterol, HDL cholesterol, LDL cholesterol, triglycerides, lipoprotein distribution, the ratio of total serum cholesterol to HDL cholesterol and acid levels in the yogurt. Duncan's multiple range was used to determine significance within and between dietary periods of the same variables if analysis of variance was significant.

RESULTS & DISCUSSION

Nutrient intake

The mean dietary intake of energy, total protein, carbohydrate, total fat, saturated fatty acids, polyunsaturated fatty acids and cholesterol was not statistically different ($P>0.05$) between the usual diet and the three yogurt diets (Table 2). As expected, mean body weight did not change significantly since energy intake did not change. Intake of calcium, phosphorus, potassium and riboflavin increased significantly ($P<0.05$) during yogurt diets. Crude fiber intake during period SH-III was 2.48 g/day below the crude fiber intake during the free choice period ($P<0.05$). Although animal protein intake increased significantly during yogurt consumption periods, a recent critical review of the literature (Sacks et al., 1983) concluded that in humans this change has no effect on plasma cholesterol.

Serum lipids and lipoproteins

Total cholesterol. The concentrations of mean total cholesterol (TC) on day 0 were not significantly different ($P>0.05$) between yogurt diet periods (Table 3). By day 7, mean TC was reduced 10% on diets incorporating yogurts CH-I and CH-II. Individually, six of the eight subjects consuming yogurt CH-I and eight of the ten subjects consuming yogurt CH-II responded with a decrease in TC. After 14 days of diet CH-I, mean TC was not significantly different from day 0, since TC in six subjects increased from day 7. From day 7 to day 14 of diet CH-II, mean TC remained unchanged although TC increased in six subjects and decreased in four subjects. From day 0 to day 7 on diet SH-III mean TC was not changed, but by day 14 mean TC had fallen 12% ($P<0.05$). Mean TC did not change from day 14 to day 21.

The results of this experiment suggest yogurt consumption by human males will lower total serum cholesterol but the hypocholesterolemic effect is not maintained by continued yogurt consumption. Total serum cholesterol was reduced 10–12% within 7–14 days and returned to control levels a week later.

These results generally agree with the findings of Mann and Spoerry (1974), Mann (1977a), and Hepner et al. (1979), who have also found that total serum cholesterol is generally reduced by yogurt consumption although the control levels have always been regained. Although Rossouw et al. (1981) concluded that yogurt was hypercholesterolemic, his results show that 3 wk of yogurt supplementation reduced total serum cholesterol 5%, even though total fat intake increased 25 g/day. Previous research from our laboratory (Massey, 1984) found no significant influence of 480 mL daily yogurt supplementation on human female serum lipids.

High-density lipoprotein cholesterol. The high density lipoprotein cholesterol (HDL-C) remained unchanged through diet periods CH-I and SH-III, although on day 7 of CH-II a 30% decline was observed ($P<0.05$). Individual HDL-C responses were sim-

Table 1—Composition of yogurts

Yogurt culture	Nutrient (g/dL)			Acidity		Acid concentration, ppm		
	Protein	Fat	Carbohydrate	pH	Titratable acidity (%)	HMG-like	Orotic	Uric
CH-I	4.0	0.0	6.1	4.20	1.13	180 ± 7	23.2 ± 1.3	16.8 ± 0.4
CH-II	3.8	0.0	6.4	4.34	1.12	156 ± 53	17.3 ± 1.2	15.2 ± 0.6
SH-IIIA	4.2	0.0	7.0	4.65	0.90	115 ± 1	28.2 ± 0.8	18.2 ± 1.2
SH-IIIB	3.9	0.0	6.9	4.46	0.98	—	—	—

*Table 2—Nutrient intake of adult males consuming usual and yogurt diets**

Diet period	Nutrients								
	Energy (kcal)	Protein (g)	Animal Protein (g)	Carbohydrate (g)	Fat (g)	Saturated fatty acids (g)	Cholesterol (mg)	Fiber (g)	Calcium (mg)
Usual n = 10	2365 ± 127	95.1 ± 7.3	35.7[a,b,c] ± 3.2	284.4 ± 12.3	91.1 ± 7.4	26.8 ± 2.1	395.5 ± 54.2	6.98[d] ± 0.71	1110.8[e,f,g] ± 124.8
CH-I n = 8	2407 ± 175	104.9 ± 7.9	65.2[a] ± 4.2	287.3 ± 15.1	93.4 ± 2.6	25.4 ± 12.2	291.0 ± 51.6	5.80 ± 0.73	1989.5[e] ± 96.9
CH-II n = 10	2464 ± 207	109.8 ± 5.9	58.7[b] ± 2.9	271.6 ± 18.3	101.5 ± 15.9	31.8 ± 6.3	346.9 ± 50.5	5.90 ± 0.79	1991.9[f] 112.2
SH-III n = 10	2468 ± 197	107.4 ± 7.8	57.7[c] ± 3.1	299.2 ± 23.7	92.7 ± 10.1	25.6 ± 3.1	337.6 ± 58.3	4.50[d] ± 0.39	1991.9[g] ± 125.3

* Values are group mean and SEM calculated from individual four day mean intake.
[a-g] Means with the same superscript are significantly different (p<0.05).

*Table 3—Serum lipid concentration of adult males consuming usual and yogurt diets**

Diet period (days consumed)	Total cholesterol (mg/dL)	HDL cholesterol (mg/dL)	Total-cholesterol: HDL-cholesterol	LDL cholesterol (mg/dL)	Total triglycerides (mg/dL)
CH-I, n = 8					
0 (Usual)	189 ± 14	44 ± 4	4.5 ± 0.47	121 ± 13	116 ± 32
7	167 ± 9[a]	41 ± 3	4.2 ± 0.39	106 ± 10	104 ± 15
14	178 ± 13	43 ± 4	4.3 ± 0.43	115 ± 13	100 ± 27
CH-II, n = 10					
0 (Usual)	182 ± 8	48 ± 3	3.9 ± 0.33	113 ± 10	103 ± 18
7	163 ± 8[a,c]	33 ± 3[a,c]	5.2 ± 0.42[a,c]	113 ± 7	83 ± 9
14	165 ± 7[a]	43 ± 3[b,d]	4.2 ± 0.22[c,d]	94 ± 5[b,c,d]	140 ± 34[a,d]
SH-III, n = 10					
0 (Usual)	178 ± 8	42 ± 3	4.4 ± 0.33	118 ± 7	90 ± 14
7	173 ± 8[c]	41 ± 3	4.4 ± 0.31	114 ± 7	92 ± 9
14	156 ± 9[a,b,d]	42 ± 3	3.9 ± 0.30[b]	95 ± 8[b,d]	96 ± 11
21, n = 8	158 ± 10[a]	39 ± 2	4.1 ± 0.32[d]	101 ± 10	86 ± 8

* Values are group mean ± SEM.
[a] Significantly different (P<0.05) from day 0 within same diet period.
[b] Significantly different (P<0.05) from day 7 within same diet period.
[c] Significantly different (P<0.05) same day between diet periods.
[d] Significantly different (P<0.05) from day 7 between diet periods.

ilar to the mean HDL-C response. Keim et al. (1981) found that a high calcium intake without yogurt or milk product supplementation reduced HDL cholesterol. In our experiment, increased calcium intake can not be associated with the reduced HDL cholesterol, since calcium intake during CH-II (2000 mg/day) was no different from the intake during CH-I and SH-III. Rossouw et al. (1981) also found that HDL cholesterol decreased during both a baseline diet period and after seven days of milk supplementation.

Although high density lipoprotein levels may be increased by intense physical activity (Hullunen et al., 1979), subjects reported no change in physical activity.

Low-density lipoprotein cholesterol. Mean low-density lipoprotein cholesterol (LDL-C) was insignificantly reduced on the CH-I diet (Table 3). On diet CH-II, LDL-C dropped 19 mg/dL after 14 days (P<0.05). After day 14 of SH-III, LDL-C decreased 23 mg/dL, although by day 21 mean LDL-C was not significantly different from control level.

Serum LDL cholesterol generally followed the response of total serum cholesterol. Keim et al. (1981) found a significant increase in LDL cholesterol in men supplemented with two quarts of skim milk daily. They suggest LDL changes reflect altered hepatic metabolism induced by the high influx of nutrients.

During period CH-II, mean intake of total protein, animal protein, total fat, and saturated fat was greater than during the free choice or the CH-I and SH-III diets (Table 2). Although the observed increase of these specific nutrients is not statistically significant (P>0.05), increases in protein and fat intake have been correlated with an increase of serum lipids and modification of serum lipoproteins.

Serum triglycerides. Serum triglycerides did not change significantly during any yogurt consumption periods (Table 3). Individual variability was large. Mean serum triglycerides fluctuated ±16 mg/dL on the CH-I and SH-III diets. After day 14 on the CH-II diet the mean serum triglyceride concentration was 140 mg/dL, although exclusion of one very high individual value from the mean triglyceride value would have brought the mean down to 108 mg/dL, which would not be significantly different from previous mean serum triglyceride values.

Serum lipoproteins. The lipoprotein distribution on day 0 did not change significantly between diet periods. No significant change in mean lipoprotein distribution was observed on diets CH-I and SH-III. On day 7 of diets CH-II the relative percentage of mean LDL increased 50%, while at the same time the relative percentage of VLDL and HDL decreased 50%. However, on day 14 of diet CH-II serum lipoproteins had returned to the control values.

Yogurt composition

In past experiments the yogurt composition has not been well characterized, making it difficult to compare results. Mann and Spoerry (1974) added olive oil, Tween 20, and riboflavin to the yogurt after room temperature incubation for 2–3 days. Mann (1977a) used skimmed milk, Hepner et al. (1979) used a Dannon product, while Rossouw et al. (1981) and Howard and Marks (1977) provided no information on the yogurt composition. In our previous studies (Massey, 1984) a commercially produced, plain, lowfat yogurt was used.

It is known that different culture strains, additives, and fermentation conditions produce different nutrient compositions in yogurt. In support of this idea Mann (1979) has proposed that a "milk factor" is produced by only certain bacteria. A change in these "factors" may influence the rate of total serum cholesterol response to yogurt supplementation.

The hypocholesterolemic agent has been suggested to be hydroxymethylglutarate (HMG), orotic acid, or uric acid. No experiment prior to this study has reported measuring the quantity of these agents in yogurt supplemented to human subjects. The assayed concentrations of these acids in the yogurts is listed in Table 1.

Hydroxymethylglutarate, when present in the body, may limit cholesterol synthesis by inhibiting the branch point enzyme HMG-CoA reductase. Mann (1977b) suggested HMG normally exists in yogurt and is enhanced by microbial action. Richardson (1978) debated whether sufficient HMG exists in yogurt to inhibit the HMG-CoA reductase in man. The amount found in these yogurts are well below the amounts needed to lower serum cholesterol in human studies (Lupien et al., 1979).

Orotic acid, a pyrimidine intermediate, may also inhibit the formation of cholesterol from acetate. Orotic acid is normally found in cows' milk at concentrations of 69–122 mg/L (Alm, 1982; Richardson, 1978) and in yogurt in the range 34–46 mg/L (Alm, 1982; Okonkov and Kinsella, 1969). The concentration range found in yogurt may differ with different yogurt culture strains (Haggerty et al., 1984). Since orotic acid is generally found in greater amounts in unfermented milk it would not appear orotic acid is the "milk factor" enhanced by fermentation.

Although uric acid has been reported to inhibit hepatic cholesterolgenesis *in vitro* (Ward et al., 1982), no studies have been done on its hypocholesterolemic effects when fed orally. Since high uric acid levels cause gout, such investigations are not likely to be done in humans, who cannot further metabolize uric acid.

Hepner et al. (1979) suggested that the ingestion of lactobacilli might alter the intestinal microflora, resulting in greater deconjugation of bile acids and increased fecal elimination of cholesterol. However, Robins-Browne and Levine (1981) reported that although orally fed *L. acidophilus* and *L. bulgaricus* entered the small intestine, elevated counts in jejunal fluids only persisted for 3–4 hr. Long-term survival or implantation of yogurt bacteria has not yet been convincingly demonstrated, and must be proven before yogurt bacteria themselves can be considered as a possible hypocholesterolemic agent.

In summary, the transient hypocholesterolemic effect of increasing yogurt consumption did not last for more than 14 days. The concentrations of uric, orotic and hydroxymethylglutaric-like acids in the yogurts were insufficient to account for the temporary hypocholesterolemic effects of yogurt consumption.

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Proximate Composition, Cholesterol, and Calcium Content in Mechanically Separated Fish Flesh From Three Species of the Gadidae Family

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ABSTRACT

Minced fish flesh, as well as the intact fillet, from three species of the *Gadidae* family were analyzed for proximate composition, cholesterol, and calcium content. Minced fish meat produced from commercially scaled whiting had elevated levels of calcium (about 0.11%) over levels found in filleted flesh (0.03%). Hand scaling prior to mincing brought the calcium content close to levels found in skinless fillets. The minced flesh for all species had the following ranges of values for proximate composition expressed as percent of wet weight: protein, 14-17%; fat, 0.4-2.0%; moisture, 81-84%; ash, 0.5-1.9%.

INTRODUCTION

A MAJOR PROBLEM for fish utilization is waste. Fillets and steaks account for only 30% of the valued landed catch, leaving 70% as carcasses to be turned into fish meal or pet food. Even more fish are discarded because they have no market value. It is estimated that an additional 20% yield could be added to the amount available as human foodstuff through the use of mechanical deboners as are used in the beef and poultry industry (King and Carver, 1970; Martin, 1976; Regenstein, 1980). It is expected that the National Marine Fisheries Service will petition the USDA to amend the Standards and Requirements for Mechanically Separated (Species) and Products in Which it is Used (USDA, 1982) to allow the inclusion of mechanically separated fish into sausage and sausage-like products, but not before the wholesomeness of the minced fish flesh is ascertained. The Standards for beef, veal, pork, and poultry define two categories of mechanically separated products; one which meets the requirements of 30% maximum fat and 14% minimum protein and the second category, in which there are no fat or protein requirements. Products in the first category can be used in any allowable meat food product. Products in the second category can only be used in meat food products that are subject to regulatory definition and standard which limits fat content. The extent to which the research conducted on mechanically deboned beef (Froning, 1976), veal (Young et al., 1983), and/or poultry (Field, 1976) can be applied to mechanically separated fish flesh or minced fish meat (MFM), as it is called, is not known.

Federal regulations specify that the separated product cannot have calcium levels exceeding 0.75%. Mechanically separated (MS) poultry and beef flesh have levels of calcium substantially greater than in the poultry/beef not mechanically minced (Field, 1976), because the bones are crushed prior to mechanical deboning. In the case of fish mince, however, it was postulated that the calcium content would not be significantly different from hand-filleted flesh, because the bones are not crushed prior to mechanical separation. Adu et al. (1983) reported calcium values of 0.05, 0.09, and 0.11% for rockfish, (*Sebastes, spp*), for hand-filleted fillets, unwashed mince from cleaned headed and gutted (H & G) fish, and washed mince from cleaned H & G fish, respectively. They felt that elevated levels of some of the minerals, not specifically calcium, found in the washed mince were due to the quality of the wash water and the equipment used in washing. Crawford et al. (1972) found increased calcium levels in mince made from six species of finfish, and values for cod, (*Gadus macrocephalus*), of 0.83, 0.11, and 1.54% for mince made from milled whole waste, flesh separated from carcass, and milled residual skin and bones from the separation process, respectively. There was, however, no comparison of calcium levels between the minced flesh from the mechanical deboning and that of hand-filleted flesh.

Cholesterol levels in MS beef, poultry, and veal were higher than in the ground product presumably because of the inclusion of bone marrow and spinal cord in the MS products (Arasu et al., 1981; Kunsman and Field, 1976; Kolbye et al., 1977; Kunsman et al., 1981; Young et al., 1983). The MFM was analyzed to determine if the same would hold true for fish.

An increased seafood yield of 20% would be worthless if there were no assurance of a consistent product of uniform quality for use as an end product or in combination with other ingredients such as may be used to make a sausage product. There were, therefore, four objectives to this study: (1) to compare the chemical composition of commercially prepared MFM to fillet flesh; (2) to determine if additional cleaning steps prior to mincing would affect the chemical composition of the MFM; (3) to determine if the calcium levels could conform to the regulations governing beef and poultry; and (4) to see if cholesterol levels were elevated in the minced product as was found in other MS meat.

MATERIALS & METHODS

THREE SPECIES of the family *Gadidae* were used for this investigation: Atlantic cod (*Gadus morhua*), silver hake (*Merluccius bilinearis*), and red hake (*Urophycis chuss*). These species are the most likely to be used in the northeast U.S. for the production of minced fish flesh. All fish were landed in November or December in the northwest Atlantic Ocean. All raw materials were minced in a single pass through a Yanagiya Meat/Bone Separator using a stainless steel drum with 5 mm diameter openings.

The Atlantic cod (300 lb) were eviscerated at sea and were about 7 days post-catch when received in the laboratory. They were hand-filleted leaving belly flaps and pin bones with the fillet (Fig. 1 and 2). The belly flaps were cut from the fillets and passed through the separator to make a mince sample from skin-on belly flaps. V-cuts were taken from the fillets and also passed through the separator to make a mince sample from skin-on V-cuts. The fillets, minus belly flaps and V-cuts, were then divided into two lots. The skin was removed from one lot of fillets, and the fillets were homogenized in a food processor to be used as the control sample. The second lot of fillets was passed through the separator with the skin side away from the drum to make a mince sample from skin-on fillets.

The frames were saved from the filleting operation. Heads only were cut from all frames. Half of the frames were put through the separator without washing making a mince sample from commercial frames. The kidney region was cut from the remaining frames, and these frames were passed through the separator making a mince sample from "cleaned" frames. The cod, therefore, yielded one control sample made from skinned fillets, and five minced samples

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made from skin-on fillets, skin-on belly flaps, skin-on V-cuts, commercial frames, and cleaned frames.

Silver hake (whiting) were examined in two separate experiments (Fig. 3). The first experiment involved 1 day post-catch fish that were commercially headed, gutted, and scaled in a processing plant and then brought to the laboratory. The fish were divided into three samples, each sample representing about 20 lb of fish. One sample was hand-filleted, skinned, and homogenized in a commercial food processor to be used as the control. The second sample was passed once through the meat/bone separator with no further processing. This represented commercially prepared mince from headed and gutted (H & G) whole fish. The third sample was thoroughly washed to remove blood, kidneys, and belly lining prior to mincing representing mince made with an additional clean-up step.

Approximately 4 day post-catch whiting were used in the second experiment. Each fish was headed, gutted, and scaled by hand in the laboratory. These hand-scaled fish were divided as described above with the resultant control (1), commercial H & G mince (2), and cleaned, H & G mince (3) as the three samples.

Two hundred pounds of red hake, 1 day post-catch, were headed, gutted, and hand scaled in the laboratory. The fish were then divided into three samples following the sampling scheme of Experiment 2 above (Fig. 3).

Moisture content was determined by drying approximately 10g of sample to constant weight at 100°C. Total ash was determined by heating dried samples to constant weight at 525°C (AOAC, 1975). Lipid content was determined on three 25g samples according to Bligh and Dyer (1959). Cholesterol was determined by the method of Kovac et al. (1979). Carbohydrate was assumed to be negligible, and protein was calculated by difference. Calcium was analyzed in triplicate by atomic absorption spectrophotometry (AOAC, 1975). Single calcium determinations were done for the second whiting experiment. Sample variation was tested as being significant at $p < 0.01$ using analysis of variance, and points of difference were detected by Duncan's (1955) multiple range test. Standard deviations for the second whiting experiment were determined by using the average of the percent of standard deviation to the mean of all samples in the range of the calcium values.

RESULTS & DISCUSSION

TABLES 1-3 SHOW proximate composition, cholesterol, and calcium data for the three species of fish. There are several different minces currently being produced from Atlantic cod, each utilizing a different portion of the cod for the raw starting material. Fig. 1 outlines the various portions that are used commercially and were examined in this experiment. Mince produced from whole, headless frames, as done commercially, was also compared to mince made from headless frames with the kidney section cut away. The mince made from both lots of cod frames was significantly higher in cholesterol and calcium than the other four lots of cod products (Table 1). Cholesterol levels nearly doubled over the skinless fillet control when using mince made from frames. Calcium levels increased almost tenfold using cleaned frames as the starting product. Mince from frames had significantly higher fat content than other cod mince.

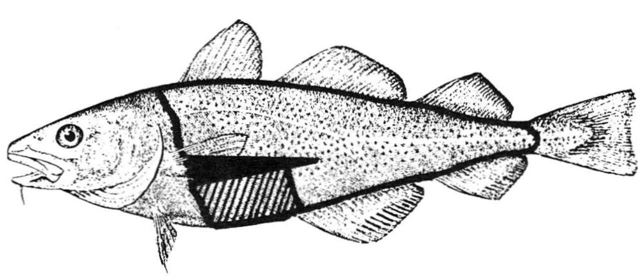

Fig. 1—Commercial cuts of Atlantic cod. Bold black lines outline a full nape cut. Black area shows the V-cut which removes the pin bones. Striped area is the belly flap. This is frequently (and incorrectly) called the nape. Full nape cut fillets minus the belly flap and V-cut are called J-cut fillets.

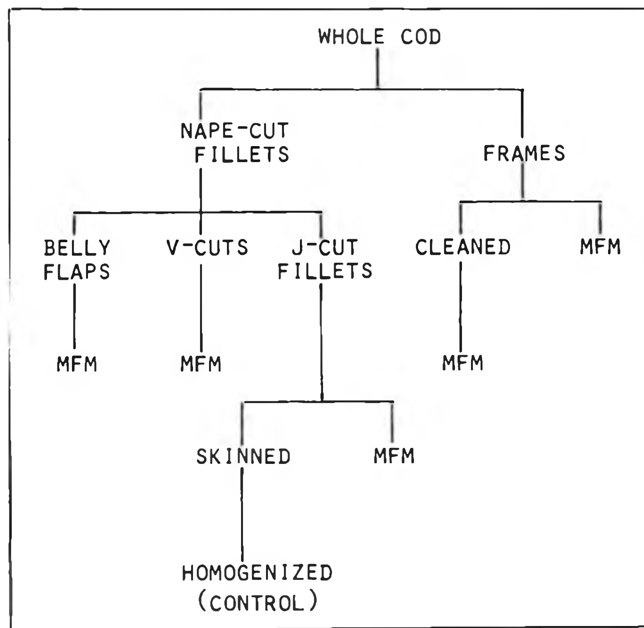

Fig. 2—Sampling scheme for Atlantic cod.

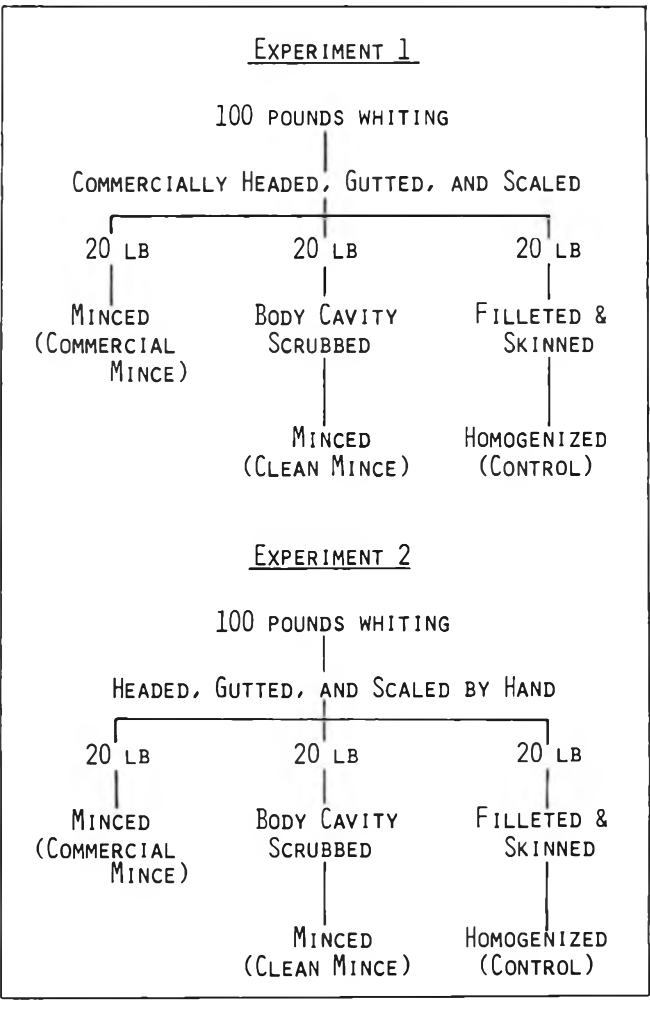

Fig. 3—Sampling scheme for whiting. Experiment 1: Commercially headed, gutted, and scaled whole fish were used as raw material. Experiment 2: Hand-scaled, headed and gutted whole fish were used as raw material. Experiment 2 also shows the sampling scheme for the red hake.

Headed and gutted whole fish are currently being used for the commercial preparation of MFM made from whiting or red hake. Any cleaning of the visceral cavity is done during the commercial scaling operation when the fish are tumbled with a high pressure water spray inside the scaling drum. An additional washing of the cavity of each fish was done in the laboratory prior to mincing to see if there were differences in proximate composition, and calcium and cholesterol levels in the MFM when employing an extra cleaning step.

The whiting (Table 2) yielded the most startling data. Each experiment on the whiting was analyzed separately for analysis of variance. Calcium values jumped from a low of about 30 mg% in the control to values of about 115 mg% made from both washed and unwashed whole fish that had been commercially scaled. The amount of calcium in the mince made from hand-scaled H & G whiting (30 mg%) was significantly lower than in the mince made from commercially scaled H & G whiting (115 mg%) and was the same as the amount found in the control (30 mg%). This suggests that the commercial scaling process did not completely remove the whiting scales, and the scales were extruded with the mince, contributing significantly to the higher calcium level in the mince. The protein content (14.3%) of the commercially scaled, washed H & G mince was close to the minimum allowable for use in sausage products, but it must be remembered that the protein content in this report was calculated by difference, and there is no standard deviation of the method, and the control samples from both whiting experiments were only about 15.0%

Table 1—Proximate composition, cholesterol, and calcium content in cod fillets and mince made from various cod portions (Percent wet weight ± one std. dev.)

Sample	% Fat	% Moisture	% Ash	% Protein	mg% Cholesterol	mg% Calcium
Skinless fillets	0.48 ± 0.05	82.4 ± 0.2	1.12 ± 0.18	16.0	48.1 ± 1.3	4.5 ± 0.6
(Control)	a,b	b,c	b		a	a
Mince:						
Skin-on fillets	0.42 ± 0.02	82.0 ± 0.3	0.89 ± 0.17	16.7	45.0 ± 5.2	14.5 ± 2.3
	a	b	a,b		a	b
V-cuts	0.55 ± 0.02	80.9 ± 0.3	1.15 ± 0.12	17.3	49.9 ± 2.8	5.3 ± 0.4
	b	a	b		a	a
Belly flaps	0.52 ± 0.04	81.7 ± 0.4	0.49 ± 0.10	17.3	55.3 ± 4.6	18.9 ± 0.9
	a,b	a,b	a		a	c
Cleaned frames	0.58 ± 0.04	83.1 ± 0.1	0.88 ± 0.30	15.4	82.9 ± 2.2	38.4 ± 0.6
	b,c	c	a,b		b	e
Commercial frames	0.66 ± 0.05	81.6 ± 0.1	1.92 ± 0.13	15.8	87.8 ± 3.2	28.6 ± 0.6
	c	a,b	c		b	d

[a] Samples with the same lower case letter are not statistically different ($p < 0.01$).

Table 2—Proximate composition, cholesterol, and calcium content of whiting fillets and mince made from H & G whiting (Percent wet weight ± one std. dev.)

Sample	% Fat	% Moisture	% Ash	% Protein	mg% Cholesterol	mg% Calcium
Commercially scaled						
Skinless fillet	1.37 ± 0.08	82.4 ± 0.05	1.17 ± 0.11	15.1	44.3 ± 1.3	29.5 ± 1.4
(Control)	a	a	a		a	a
Mince:						
Cleaned H & G	1.51 ± 0.02	82.6 ± 0.1	1.52 ± 0.06	14.3	46.8 ± 5.4	101.6 ± 1.7
	a	a	a		a	b
Commercial H & G	1.91 ± 0.42	81.9 ± 0.2	1.25 ± 0.11	14.9	68.1 ± 6.6	127.3 ± 0.6
	a	a	a		b	c
Hand-scaled						
Skinless	1.92 ± 0.08	82.0 ± 0.1	1.12 ± 0.16	15.0	42.2 ± 1.5	28.1 ± 1.8
(Control)	a	a	a		a	a
Mince:						
Cleaned H & G	1.94 ± 0.02	82.0 ± 0.1	1.11 ± 0.16	14.9	44.0 ± 7.0	31.0 ± 2.0
	a	a	a		a	a
Commercial H & G	2.00 ± 0.38	81.4 ± 0.5	1.08 ± 0.00	15.5	57.0 ± 16.7	32.0 ± 2.0
	a	a	a		a	a

[a] Samples with the same lower case letter are not statistically different ($p < 0.01$).

Table 3—Proximate composition, cholesterol, and calcium content in red hake fillets and mince made from H & G red hake (Percent wet weight ± one std. dev.)

Sample	% Fat	% Moisture	% Ash	% Protein	mg% Cholesterol	mg% Calcium
Skinless fillets	0.36 ± 0.01	84.5 ± 0.2	1.07 ± 0.01	14.1	35.1 ± 2.2	20.4 ± 0.7
(Control)	a	a	a		a	a,b
Mince:						
Cleaned H & G	0.45 ± 0.03	83.2 ± 0.3	1.58 ± 0.24	14.8	49.0 ± 2.5	25.5 ± 1.0
	a	a	a		a	b
Commercial H & G	0.37 ± 0.05	83.1 ± 0.4	1.30 ± 0.12	15.2	45.3 ± 6.4	20.0 ± 1.2
	a	a	a		a	a

[a] Samples with the same lower case letter are not statistically different ($p < 0.01$).

protein. Brooke et al. (1962) reported a protein content of 15.2 ± 0.9% for silver hake caught in the fall. This means that the range of protein for whiting at two standard deviations is 13.4–17.0%. Whiting may produce a mince that cannot pass the minimum requirements.

In the case of red hake (Table 3), fat moisture, ash, and cholesterol content remained the same for all samples. While the control had protein levels (14.1%) approaching the minimum allowable (14.0%) under the regulations, the protein content in the MFM did fall within the acceptable guidelines at 14.8 and 15.2%. Sidwell (1981) reported a range for protein in the raw muscle of *Urophycis chuss* of 15.2–20.7%.

When comparing all three species and their various MFM, cholesterol levels were the highest in the cod frame minces at about 85 mg/100g mince. The cod frame minces had relatively low fat content at about 0.6%, while some of the highest fat samples, the whiting minces, at about 1.9% fat, were significantly lower in cholesterol at about 40 mg/100g.

The H & G commercially scaled whiting mince contained significantly higher amounts of calcium, more so than the cod which were never scaled commercially or by hand. Cod scales were therefore, either not extruded with the MFM because of their size, adhesion to the skin, or the position of the skin to the drum (Wong et al., 1978), or, the scales were extruded but were not as numerous as the scales on the whiting and did not constitute a major weight percentage of the sample. The cod samples, as a group, had the lowest levels of calcium followed by the red hake.

The following generalizations were made with the MS beef/poultry regulations as the standard for fat, protein, and calcium. Cholesterol evaluations were based on the American Medical Association's recommendation of 300 mg cholesterol/day for people on low-cholesterol diets. The red hake mince was low fat, low cholesterol, low calcium, and acceptable protein. The red hake was difficult to process, because hand-scaling was involved. The whiting mince was low fat, low to medium cholesterol, low calcium (if properly scaled), and perhaps marginal protein. Once, again, hand-scaling made the processing tedious. Cod mince was low fat, medium to high cholesterol, low calcium, and high protein. Cod was the easiest to process of the three species tested. It would appear that mechanical separating did not substantially alter the chemical composition of the MFM from that of the skinless fillet. Protein content may become a problem if these species do not have an initial protein content of 14.0% before they are even processed.

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Mention of a trade name or particular product does not constitute endorsement by the NOAA to the exclusion of other products that may also be suitable.

Use of Fluorogenic Assays for the Enumeration of *Escherichia coli* from Selected Seafoods

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ABSTRACT

Escherichia coli was enumerated and immediately confirmed by incorporating 4-methylumbelliferone glucuronide (MUG) into Lactose Broth (LB), Violet Red Bile Agar (VRB) and M-Endo Broth (M-Endo) in selected seafoods. *E. coli* in the seafood samples ranged from $1.0 \times 10^1 - 1.0 \times 10^3$ Most Probable Number (MPN)/g, $1.0 \times 10^1 - 3.5 \times 10^2$ cfu/g and $0.9 \times 10^1 - 2.8 \times 10^2$ cfu/g in LB-MUG, VRB-MUG, and M-Endo-MUG, respectively. Although not statistically significant ($\alpha = 0.05$), LB-MUG resulted in higher counts in fresh finfish, frozen fish and breaded seafoods. Specificity for *E. coli* in LB-MUG, VRB-MUG and M-Endo MUG was 96.5%, 92.0%, and 90.0%, respectively. All three fluorogenic assays enabled a rapid and sensitive enumeration of *E. coli* from seafood products.

INTRODUCTION

THE OCCURRENCE of fecal coliforms in foods is regarded as an important indication of public health hazard from infectious agents. Coliforms, fecal coliforms, fecal streptococci, *Escherichia coli,* and enteropathogenic/enterotoxigenic *E. coli* have all been used as sanitary indicators of fecal pollution in foods and water (Chordash and Insalata, 1978). *E. coli* is often preferred over the coliform group as an indicator because it is more specific and more reliably reflects fecal origin (Feng and Hartman, 1982). Current detection assays for *E. coli* are based on the properties of acid or gas production from lactose. Some of the accepted methods include the membrane filter method, the Most-Probable-Number (MPN) method involving a presumptive test followed by a confirmed test, and the direct pour plate method using Violet Red Bile (VRB) agar. All these tests are laborious and time consuming, some requiring 48 - 96 hr to complete (Speck, 1976).

The use of the fluorogenic substrate (4-methylumbelliferone-β-D-glucuronide (MUG) to detect glucuronidase activity was first reported by Mead et al. (1955). In 1976, Kilian and Bülow reported glycosidase activities among *Enterobacteriaceae* and demonstrated that β-glucuronidase activity was an exclusive characteristic of *E. coli* and some *Shigella* species. Among 633 *Enterobacteriaceae,* 97% of the *E. coli* and 50% of the *Shigella* tested were positive for glucuronidase activity; all other *Enterobacteriacea* were negative. LeMinor (1978) found that of 4000 strains of *Salmonella* tested, 30% possessed glucuronidase. Kilian and Bülow (1976) concluded that the high frequency observed among *E. coli* strains indicated that the β-glucoronidase presence had potential for simplifying the differentiation of *E. coli* from other enterobacteria.

Kilian and Bülow (1979) formulated an agar medium (PGUA-Agar) which permitted the detection of bacteria with β-glucuronidase activity in mixed cultures using a chromogenic substrate, 4-nitrophenyl-β-D-glucuronide, as the substrate. Other researchers have studied β-glucuronidase activity (Buehler et al., 1951; Doyle et al., 1955; Kushinsky et al., 1967). Feng and Hartman (1982) developed a rapid assay for *E. coli* in Most-Probable-Number tubes by adding 100 μg/mL of MUG into Lauryl Sulfate Tryptose Broth (LST). Approximately 90% of the tubes showing both gas production and fluorescence contained fecal coliforms (positive in EC broth incubated at 45°C). The authors described the procedure as being sensitive and rapid.

Recently, Firstenberg-Eden and Klein (1983) reported a new medium developed for the impedimetric detection of coliforms. The selectivity of CM medium was better than that of LST and VRB. However, the procedure described required a BACTOMETER M120.

The seafood industry could use a rapid (less than 24 hr) method for the detection of fecal pollution. The present study was undertaken in order to adapt fluorogenic assays for the enumeration and confirmation of fecal contamination (especially *E. coli*) from seafood products.

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MATERIALS & METHODS

Samples

Seafood samples were obtained from local retail seafood markets in the Madison, WI area. Fresh finfish, fresh shellfish, frozen fish, frozen shellfish and breaded seafoods were bought and transported to the laboratory. Samples were tested the same day of purchase.

Media and reagents

Lactose Broth (LB), Violet Red Bile Agar (VRB), M-Endo Broth, EC Broth and Eosin Methylene Blue (EMB) were all obtained from GIBCO Laboratories (Madison, WI). 4-methylubelliferone-β-D-glucuronide (MUG) was purchased from Sigma Chemical, St. Louis, MO.

Analyses of seafood samples

Seafood samples were blended in a Waring Blendor at low speed for 2 min. Appropriate serial dilutions were made by using Buttersfield Phosphate Buffer (BAM, 1976). MUG (100 μg/mL) was added to each medium (Feng and Hartman, 1981). With the MPN method (Speck, 1976), MUG was added directly to Lactose Broth (LB-MUG). The LB-MUG medium was then dispersed into tubes containing Durham vials, and the tubes sterilized in an autoclave following manufacturers specifications. The presence of *E. coli* was reflected by the appearance of fluorescence throughout the tube. The LB-MUG combination provides a presumptive coliform enumeration based on gas formation, then provides an immediate confirmation of *E. coli* based on fluorescence. With the VRB method (Speck, 1976), MUG was incorporated into the VRB overlay (VRB-MUG). *E. coli* colonies were readily distinguished from non-*E. coli* colonies by the presence of a fluorescent halo around the colonies. MUG was dissolved in warm water, filter sterilized through a 0.22-μm membrane and added to the sterilized M-Endo-MUG) (Speck, 1976). Seafood samples were filtered through a 0.45-μm membrane and the filter was then placed on the M-Endo-MUG broth plates. *E. coli* appeared as fluorescent colonies on the membrane filter.

Plates and tubes were incubated overnight at 35°C and then examined for fluorescence under long wave UV light (Blacklight blue, Westinghouse, Bloomfield, NJ).

Selected positive tubes and colonies were confirmed for *E. coli* by inoculating into an EC Broth tube incubated at 45.5°C for 48 hr followed by streaking onto an EMB plate incubated for 24 - 48 hr at 35°C (Speck, 1976). Traditional *E. coli* confirmatory steps were then performed (Speck, 1976).

Table 1—Enumeration of total cloiforms (gas) and E. coli (fluorescence) present in seafood samples using the LB-MUG assay

Samples	No. of Samples	LB-MUG[a]			
		Gas	(%)[b]	Fluorescence	(%)[c]
Fresh finfish	5	4.5×10^2	(100)	8.0×10^1	(17.7)
Fresh shellfish	4	5.8×10^2	(100)	1.6×10^1	(2.7)
Frozen fish	7	2.5×10^2	(100)	2.6×10^1	(10.4)
Frozen shellfish	5	3.5×10^2	(100)	1.0×10^1	(2.8)
Breaded seafoods	9	3.9×10^3	(100)	1.0×10^3	(25.6)

[a] Average of four replicates.
[b] Represents the Gas (+) LB-Mug tubes, the total coliform fraction in each food category.
[c] Represents the fraction of fecal coliforms present in each seafood category.

Table 2—EC broth E. coli confirmatory results on LB-MUG gas (+) tubes obtained from the MPN analysis of seafood samples

Sample	Fluorescence	No. tested	Positive EC broth test	%
Fresh Finfish	+	87	87	(100)
	–	32	0	(0)
Fresh Shellfish	+	94	90	(96)
	–	26	2	(8)
Frozen Fish	+	80	79	(97)
	–	29	1	(3)
Frozen Shellfish	+	77	77	(100)
	–	22	0	(0)
Breaded Seafoods	+	118	107	(91)
	–	30	2	(7)
TOTAL	Gas (+) Fluorescence (+)	456	440	(96.5)
	Gas (+) Fluorescence (–)	139	5	(3.5)

Table 3—Enumeration of total coliforms and E. coli (fluorescent halo) present in seafood samples using the VRB-MUG assay

Samples	No. of samples	VRB-MUG[a]		
		Total	Fluorescent halo	(%)[b]
Fresh finfish	5	4.5×10^2	2.0×10^1	(7.1)
Fresh shellfish	4	4.6×10^2	2.8×10^1	(6.1)
Frozen fish	7	1.0×10^2	1.0×10^1	(1.0)
Frozen shellfish	5	2.8×10^2	1.2×10^1	(4.3)
Breaded seafood	9	2.8×10^3	3.5×10^2	(12.5)

[a] Average of four replicates.
[b] Represents the fraction of fecal coliforms present in each seafood category.

Table 4—EC broth E. coli confirmatory results of VRB-MUG colonies isolated from seafood samples

Sample	Fluorescent halo	Colonies		
		No. tested	Positive EC broth test	(%)
Fresh finfish	+	10	10	(100)
	–	10	0	(0)
Fresh shellfish	+	10	9	(90)
	–	10	1	(10)
Frozen fish	+	8	6	(75)
	–	10	1	(10)
Frozen shellfish	+	10	9	(90)
	–	10	0	(0)
Breaded seafoods	+	25	24	(96)
	–	10	2	(20)
TOTAL	Fluorescent halo (+)	63	58	(92)
	Fluorescent halo (–)	50	4	(8)

Table 5—Enumeration of total coliforms and E. coli (fluorescent colonies) present in seafood samples using the M-Endo-MUG assay

Samples	No. of samples	M-Endo-MUG[a]		
		Total colonies	Fluorescent colonies	(%)[b]
Fresh finfish	5	2.5×10^2	1.5×10^1	(6.0)
Fresh shellfish	4	4.0×10^2	1.5×10^1	(3.8)
Frozen fish	7	1.0×10^2	0.9×10^1	(0.9)
Frozen shellfish	5	2.5×10^2	1.0×10^1	(4.0)
Breaded seafoods	9	2.2×10^3	2.8×10^2	(12.7)

[a] Average of four replicates.
[b] Represents the fraction of fecal coliforms present in each seafood category.

Table 6—EC broth E. coli confirmatory results of M-Endo-MUG colonies isolated from seafood samples

Sample	Fluorescence	Colonies		
		No. tested	Positive EC broth test	(%)
Fresh finfish	+	10	90	(90)
	–	10	1	(10)
Fresh shellfish	+	10	9	(90)
	–	10	0	(0)
Frozen fish	+	8	8	(100)
	–	10	0	(0)
Frozen shellfish	+	9	8	(89)
	–	10	1	(10)
Breaded seafoods	+	25	22	(88)
	–	10	3	(30)
Total	Fluorescence (+)	62	56	(90)
	Fluorescence (–)	50	5	(10)

The data were analyzed by two-way analysis of variance calculations (Steele and Torrie, 1960) and Duncan's multipel range test (Duncan, 1955).

RESULTS & DISCUSSION

TABLES 1, 3 AND 5 show the presence of total coliforms and *E. coli* in fresh and frozen fish and shellfish and breaded seafoods as enumerated by using LB-MUG, VRB-MUG, and M-Endo-MUG. Each count represents the mean of four replicates of each sample within each seafood category and the percent containing *E. coli* (fluorescence) were determined in relation to total coliforms (non-fluorescent colonies or gas production only). Overall, the number of *E. coli* present in the selected seafood samples are within the ranges shown in the literature (Jay, 1978). Although not statistically significant ($\alpha = 0.05$ level), LB-MUG media recovered more *E. coli* in fresh finfish, forzen fish, and breaded seafoods. Typically, the *E. coli* recovery on LB-MUG was two to three times higher in these seafood samples. Wehr (1982) reported that 29 state health and agricultural agencies had implemented policies or micorbial criteria for foods. Of these, 15 listed some criteria for seafood products. For the seafood samples with a given guideline, the coliform levels found in this study are generally higher. Wehr (1982) also stated that 7% of the survey respondants believed the presence or absence of *E. coli* was indicative of product safety. The highest percentage of *E. coli* was present in breaded seafood products (25.6%, 12.5%, and 12.7% for LB-MUG, VRB-MUG and M-Endo-MUG, respectively).

The EC Broth confirmtory test results for the selected seafood samples are shown in Tables 2, 4, and 6. Of a total of 456 gas (+) fluorescence (+) LB-MUG tubes, 440 had positive EC Broth tests (96.5%). A low-false positive rate of 3.5% was observed (positive EC test in the absence of fluorescence). No gas (–) fluorescent (+) tubes were seen in the seafood samples (Table 2). Of a total of 63 colonies with fluorescent halos, 58 had positive EC Broth tests (92.0%). A false-positive rate of 8.0% was observed (Table

—*Continued on page 1232*

Life Cycle of Canned Tomato Paste: Correlation Between Sensory and Instrumental Testing Methods

JEAN R. ECKERLE, CHARLOTTE D. HARVEY, and TUNG-SHAN CHEN

ABSTRACT

Canned tomato paste was examined to determine its life cycle. Life cycle information can be used to predict the shelf-life of the product. The product was stored at 45, 37.8, 29.4, 22.2, and 3.3°C for up to 3 yr. The product was analyzed by two sensory testing methods (hedonic evaluations and descriptive profile analysis) and three instrumental testing methods (serum color, hydroxymethylfurfural content and colorimeter values). Two separate studies were conducted to check the repeatability of the data. Correlations were established between the sensory and instrumental methods. The product had a Q_{10} of 3.8 with a shelf-life of 38 months at 25°C and an E_a (activation energy) of 23 Kcal/mole, as determined by the descriptive profile analysis.

INTRODUCTION

SURVEYS have shown that the consumer wants open code dating or shelf-life dating which is mandatory in 21 of the states in the U.S. [Labuza, 1982; OTA (Office of Technology Assessment), 1979]. The Institute of Food Technologists' expert panel on food safety and nutrition defined shelf-life as the period between manufacture and retail purchase of a food product during which the product is of satisfactory quality (Anonymous, 1974). The life cycle of a product begins at manufacture and ends when the product is totally unacceptable. Therefore, the life cycle is longer than the shelf-life of a product. They both provide information on a product's rate of deterioration under various environmental conditions. Life cycle information can assist food manufacturers in producing and distributing products of acceptable quality to the consumer.

There is no uniform shelf-life dating system used in the U.S. today (Labuza, 1982). The Office of Technology Assessment (OTA, 1979) stated that sensory quality is the most important criterion for establishing open code dating on nonperishable foods. Studies in the 1960's employed preference testing as the sensory evaluation method to establish shelf-life (Cecil and Woodroof, 1962; Peryam, 1964). The American Society for Testing and Materials (ASTM, 1979) recommends an analytical sensory method using a trained profile panel (TPP), either discriminatory or descriptive, for shelf-life testing. Swift and Company has established numerous TPP that are product specialized (Dethmers, 1979) and similar panels were used at the Stanford Research Institute (Stone et al., 1974). However, sensory testing is usually expensive and time consuming and compliance testing from a sensory standpoint would be extremely difficult. It would be highly desirable if an instrumental testing method that is highly correlated with the appropriate sensory testing could be used to replace or supplement the sensory panel (Levitt, 1974; Powers, 1974).

The major chemical deterioration that occurs in canned tomato paste can be monitored by measuring the accumulation of hydroxymethylfurfural (HMF). HMF is an intermediate in the Maillard or nonenzymatic browning reaction, resulting in the darkening of the serum color (Luh et al., 1958; 1964). The darkening of the serum color can be measured spectrophotometrically. Hernandez and Feaster (1960) claimed that an experienced taste panel was capable of detecting differences in the flavors between two reconstituted tomato juice samples varying 0.15 in spectrophotometric absorbance readings in serum color and that samples with a serum color above 0.70 in absorbance were objectionable from a flavor standpoint. These nonenzymatic browning reactions follow zero order kinetics (OTA, 1979).

The purpose of this study was to determine if a significant correlation may exist between a sensory and an instrumental testing method used to determine the effect of storage temperature on the rate of quality deterioration of canned tomato paste.

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MATERIALS & METHODS

Material

A production lot of Contadina tomato paste in 12 oz cans was obtained from the 1979 and again from the 1980 pack season. The 1980 pack season product was used to check the repeatability of the results derived from the 1979 pack season study. The cans were stored in constant (±2°C) environmental chambers at 45, 37.8, 29.4, and 22.2°C for up to 3 yr. An additional chamber of 3.3°C was used to store the control samples which remained essentially unchanged throughout the study. These temperatures were chosen because they were the only ones available at the research facility. Samples were taken from each chamber for analysis at various time intervals.

Sensory methods

Hedonic evaluation. A taste panel consisting of 24 employees of the Carnation Research Laboratory (CRL) evaluated the flavor of the coded test samples along with the blind control on a 9-point hedonic scale (1 = dislike extremely, and 9 = like extremely). Since the results from the 1979 study indicated that the trained profile panel provided sufficient information on the rate of deterioration on the product, hedonic evaluations were not used in the 1980 study due to the high cost of sensory testing.

Trained profile panel (TPP) evaluation. A descriptive analysis flavor profile panel consisting of 5-6 trained CRL employees evaluated the coded test samples along with the blind control for product acceptability (PAT) score on a 5-point scale (1 = excellent or acceptable, 2 = good or acceptable with some off notes or intensities, 3 = fair or acceptable-quesionable, 4 = poor or unacceptable-questionable and 5 = very poor or definitely unacceptable) for color, aroma and flavor-by-mouth (FBM) and intensities of perceived flavor notes. The panel members were trained to describe character changes in tomato products. When a test sample reached an average PAT of 4.5 or above in FBM, it was considered to be unsuitable for further testing and, therefore, to have reached the end of its life cycle.

Instrumental methods

Serum color. The analysis of the serum color was performed using the method described by Luh et al. (1958) with some modifications (CRL, 1979). Tomato paste samples were first diluted with distilled water to contain 5-8% total solids. After centrifugation for 10 min at 1800 rpm the supernatant was mixed with a 10% (W/V) pectinol solution and incubated for 90 min at 40°C. The filtrate

was diluted to 2.5% total solids with distilled water and read spectrophotometrically at 420 nm.

Hydroxymethylfurfural (HMF) content. The HMF content of the tomato paste was determined by a high pressure liquid chromatography (HPLC) method developed at Carnation Research Laboratory (CRL, 1981). Samples of tomato paste were extracted with distilled water. After centrifugation for 20 min at 10,000 rpm the supernatant was filtered through a millipore filtering assembly (Gelman Metricel TCM-450 pore size 0.45 μ). The filtrate was then injected into the HPLC (Hitachi Model 100-30). The column used was a 4.6 x 250 mm Lichrosorb RP8 Helbar column. Water was used as the solvent with a flow rate of 3.0 mL/min. The UV detector at 280 nm with a sensitivity of 0.05 was used. HMF purchased from ICN Pharmaceutical, New York, was employed as a standard.

Hunter Colorimeter values. The color of the tomato paste samples was determined by a Hunter Color Meter model number D25. A red plate number D33C-1188 was used as a standard.

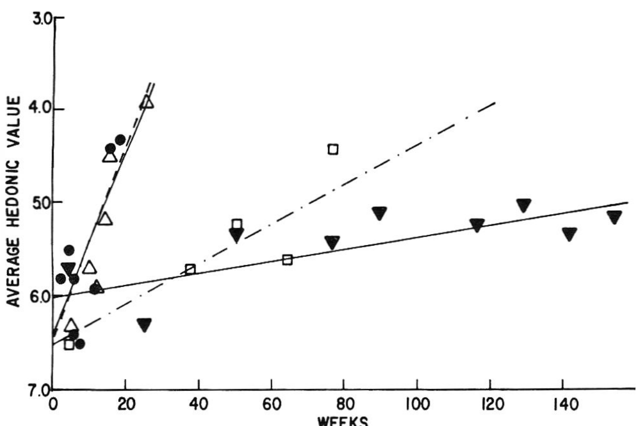

Fig. 1—Hedonic testing score vs time in the 1979 study of canned tomato paste (hedonic values range from 1, dislike extremely to 9, like extremely, n = 24): 45°C ●—●; 37.8°C △---△; 29.4°C □—·—□; and 22.2°C ▼—▼.

Statistical analysis

The sensory, PAT's and hedonic evaluation, and instrumental data for each storage temperature were fitted linearly and logarithmically with time to determine if a zero or first order reaction was taking place. The correlation coefficients were high for most of the linear regressions. Therefore, a linear relationship between quality attribute changes and storage time was assumed for all the data sets. The slopes, representing zero order rate constants, estimated from the linear regression, were used in calculating the Arrhenius relationship and the sensitivity of the reaction rate to temperature (Q_{10}).

All of the sensory and instrumental data were also analyzed by multiple regression. The following model was used in the multiple regression:

$$y = a + bw + ct + dwt + ew^2 + ft^2$$

where: y = the attribute being fitted, w = the weeks of storage, t = the temperature of storage and a, b, c, d, e, and f are constants. This quadratic model was chosen because, it is a fairly general model and it is used in other applications such as Response Surface Methodology. If the regression analysis showed a significant ($P < 0.05$) fit for a given attribute, then it was concluded that the changes in that attribute were related to the time and temperature of storage.

The data collected in the 1979 study were compared to the data collected in the 1980 study. The general linear test (Neter and Wasserman, 1974) was used to test if the quadratic equations described above were the same for each study. The general linear test uses a statistic which compares the residual sum of squares when the data for each study are fitted separately to the residual sum of squares when the data are combined. If the general linear test was significant ($P < 0.05$), then a similar analysis was performed for each temperature separately. This additional analysis again assumed a linear relationship between the quality attributes and time. Regression equations relating the quality attribute to storage time were calculated for each temperature and study. The corresponding equations for each study were tested to see if they were the same. If this test was significant ($P < 0.05$) then the slopes of the lines and the means of the quality attribute were tested to determine if they were differ-

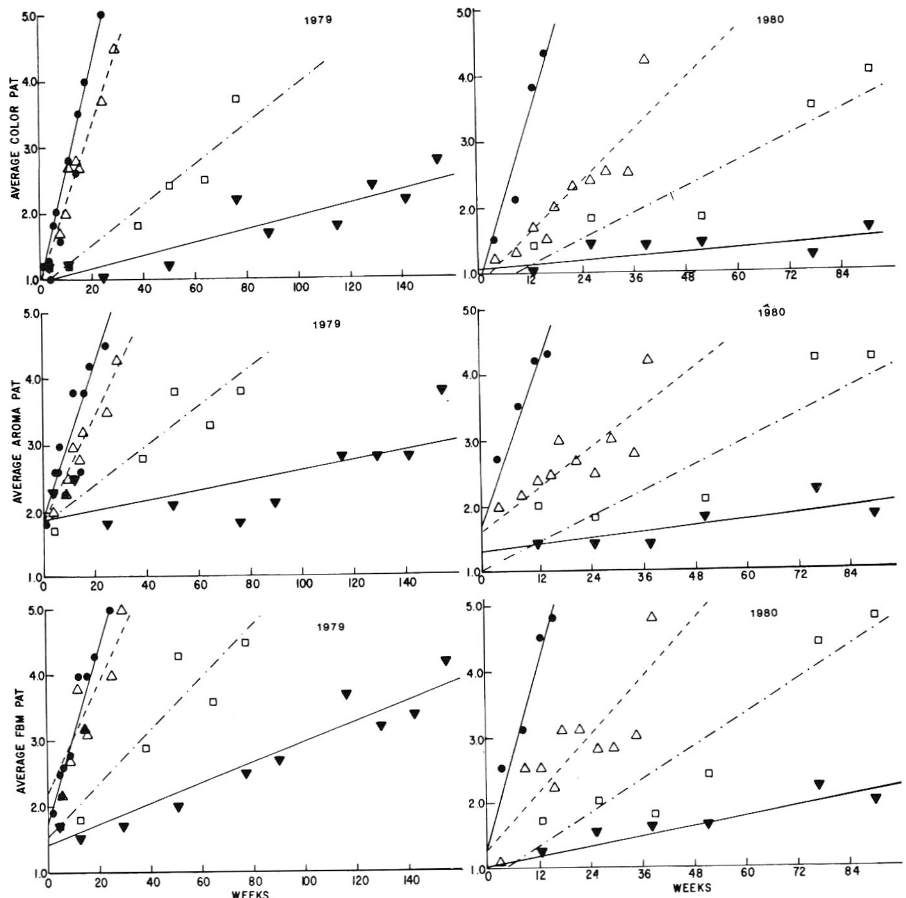

Fig. 2—Trained profile panel PAT scores vs time in the 1979 and 1980 studies of canned tomato paste (PAT = production acceptability score, values range from 1, acceptable to 5, unacceptable, n = 5-6): 45°C ●—●; 37.8°C △—·—△; 24.4°C □—·—□; and 22.2°C ▼—▼.

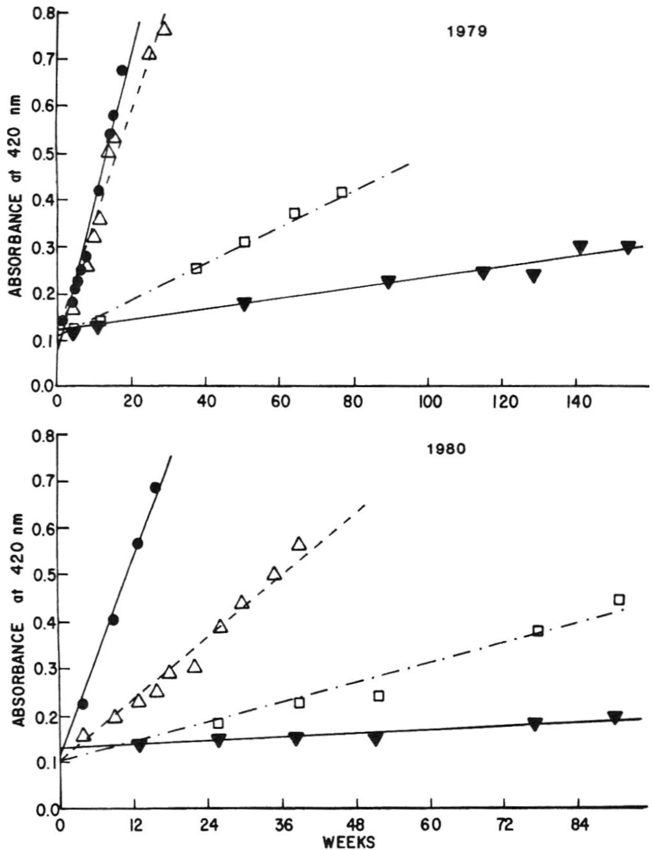

Fig. 3—Serum color values versus time in the 1979 and 1980 studies of canned tomato paste: 45°C ●—●; 37.8°C △---△; 29.4°C □—·—□; and 22.2°C ▼—▼.

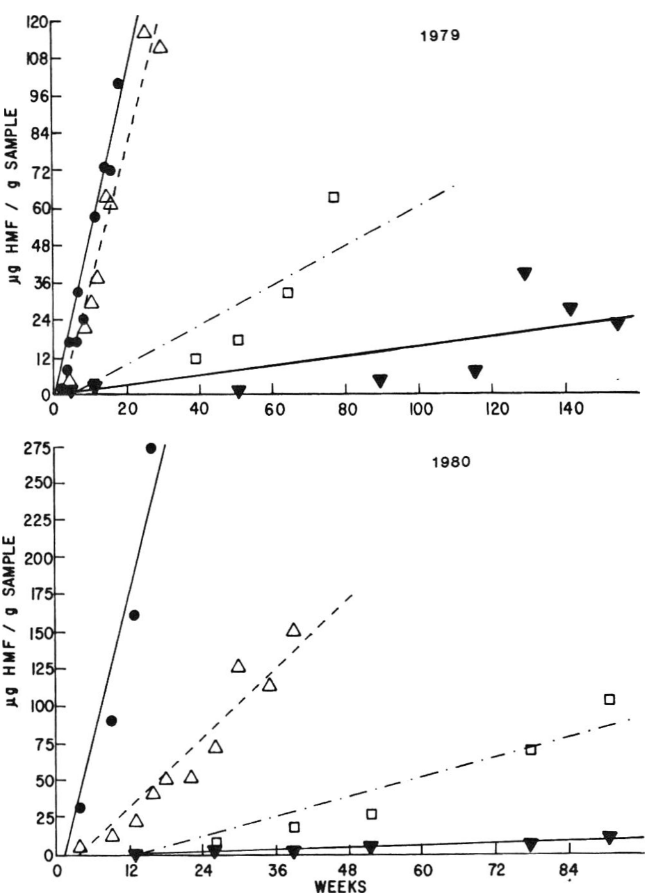

Fig. 4—Hydroxymethylfurfural content vs time in the 1979 and 1980 studies of canned tomato paste: 45°C ●—●; 37.8°C △---△; 29.4°C □—·—□; and 22.2°C ▼—▼.

ent thus indicating which storage temperatures, if any, were showing differences between the two studies (Dixon and Massey, 1968). All of the above statistical tests used the F-distribution to determine the significance of the test statistics.

RESULTS & DISCUSSION

Sensory methods

Hedonic evaluation. The decrease in the flavor hedonic score was inversely related to temperature and length of storage in the 1979 study (Fig. 1). Multiple regression analysis indicated that the decrease in the hedonic value of the tomato paste during storage was significant ($P < 0.01$). In the 1980 study, only the TPP analysis was used for sensory evaluation of the sample and the hedonic evaluation was not used in order to reduce the cost of the study.

Trained profile panel. The PAT scores were plotted versus time at the temperature employed (Fig. 2). The rate of deterioration, as indicated by the slope of the linear regression line, increased with increasing storage temperature. The product stored at 22.2°C in the 1980 study was not taken to the same end point (average PAT of 4.5 or above in FBM) as the other product due to a time limitation in the study. The correlation coefficients calculated for the regression lines were higher (0.9 or above) for the FBM data than for the aroma and color data.

The multiple regression analysis showed that all the PAT scores (aroma, color and FBM) and the following flavor notes changed significantly ($P < 0.01$) during both the 1979 and 1980 studies: aroma notes – cooked tomato, green vegetables, hay, caramelized, acrid and burnt; FBM notes – cooked tomato, bitter, hay, burnt, caramelized and sulfur-rotten tomato. The intensities of cooked tomato and green vegetable decreased as the product deteriorated; all other attributes mentioned increased. The flavor notes in the product which did not exhibit a significant change include: aroma notes – metallic, sugar bag sweet, briny, earthy vegetable and prune; FBM notes – sweet, sour, salt, metallic and prune.

Instrumental methods

Serum color. The absorbance readings at 420 nm of the tomato serum increased with time and temperature in both studies (Fig. 3). These findings agree with those of Luh et al. (1958). The linear regression calculations used to indicate the rate of deterioration of the tomato paste showed that the increase in absorbance readings at 420 nm with time was significant ($P < 0.01$) with high correlation coefficients (>0.93).

Hydroxymethylfurfural content. The HMF content increased in the tomato paste, in both the 1979 and 1980 studies, over time at each temperature examined (Fig. 4). The level of significance obtained for the linear regression calculations was 0.01. The increase in HMF content found in this study agreed with the findings of Luh et al. (1958). The higher the temperature, the faster was the rate of increase in HMF content.

Hunter Colorimeter values. The data obtained from the colorimetric analyses are presented in Fig. 5. Hunter L represents the lightness, a is the redness, and b indicates the yellowness of the paste. As the numerical values decreased, the degree of each color attribute decreased. The tomato paste became darker, less red and less yellow as the temperature of storage and length of time increased, except for

product stored at 22.2°C in the 1980 study. The linear regressions calculated for the products were found to be significant at the 0.01 level except for the product stored at 22.2°C, which did not reach the 0.05 level of significance. Luh et al. (1964) and Yeatman (1969) have demonstrated that colorimeter values can be used to indicate heat damage due to the processing conditions employed when producing tomato paste. This current study indicated that the colorimeter values can be used to indicate heat damage due to the prolonged exposure at elevated storage temperatures.

Correlation between the 1979 and 1980 studies

Instrumental methods. The general linear test indicated that most of the instrumental analytical data for the 1979 and 1980 studies were significantly different ($P < 0.01$ or < 0.05). The difference between the two studies is demonstrated in Table 1. These differences can be attributed to two factors. First, there was a problem with the 37.8°C chamber in the 1979 study: the fluctuation in temperature was greater than ±2°C. This was corrected prior to the initiation of the 1980 study. The F-statistics for the 37.8°C data were always significant ($P < 0.01$ or < 0.05) when examining the equality of the lines of the two studies. Secondly, the initial values for the instrumental analyses were different for the two studies, thus creating a significant difference in the equality of the means between the two studies. In most cases, except for the above mentioned temperature problem the F-statistics indicated the difference in the equality of the lines to be attributed to a difference in the equality of the means and not the equality of the slopes. Thus similar general responses in the instrumental analyses were taking place in both studies.

Sensory methods. The actual numerical values obtained from the TPP for most of the attributes were found to be different when the two studies were compared. This difference can be attributed to the differences in the equality of the means, as in the instrumental methods. The PAT's and flavor notes that changed during both studies were basically the same (data not shown).

Correlation between sensory and instrumental methods

The correlation coefficients in Table 2 indicate that the PAT scores and the hedonic values obtained from the sensory tests were highly correlated with the data from all the instrumental analyses used in this study. The highest correlation coefficients established were between color PAT scores and serum color ($r = 0.928$ and 0.913), HMF content ($r = 0.926$ and 0.859) and colorimeter Hunter L value ($r = -0.928$ and -0.922).

The data were also analyzed to determine if the correlations found between the sensory and instrumental testing methods were the same for both the 1979 and 1980 studies. The F-statistics (Table 2) show that the only pair of testing methods which was found to be significantly different between the 1979 and 1980 studies was the color PAT versus serum color analysis. Most of the differences in the other pairs of testing methods can be attributed to the differences in the equality of the means. This follows the same trend that was seen when analyzing the differences between the 1979 and 1980 studies for the sensory and instrumental analyses independently.

The hedonic evaluations in the 1979 study did not attain a high correlation coefficient with the PAT scores. The instrumental analyses which had the highest correlation with the hedonic evaluations were serum color ($r = 0.814$) and colorimeter Hunter a ($r = 0.859$) (Table 1). The TPP evaluations provided higher correlations with instrumental testing methods than did the hedonic test, making the TPP

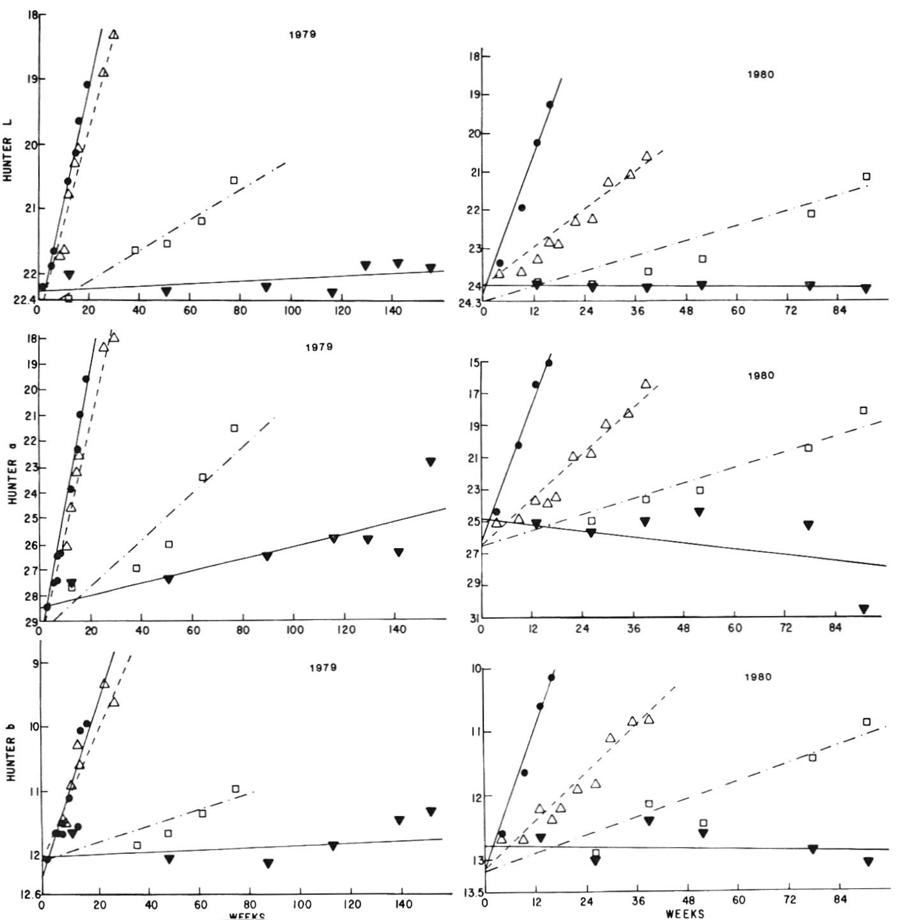

Fig. 5—Hunter colorimeter values vs time in the 1979 and 1980 studies of canned tomato paste: 45°C ●—●; 37.8°C △---△; 29.4°C □—·—□; and 22.2°C ▼—▼.

Table 1—Instrumental analyses. Statistical tests between the 1979 and 1980 study of tomato paste

Analysis	Temp °C	F-Statistic[a] Quadratic Equations	To Test for Equality of Lines	Slopes	Means
Serum Color		2.19*			
	45		16.32**	2.24	27.34**
	37.8		123.91**	57.23**	40.14**
	29.4		4.30*	0.19	9.25*
	22.2		1.17		
HMF Content		3.56**			
	45		68.39**	49.09**	16.33**
	37.8		6.29*	0.55	12.42**
	29.4		2.96		
	22.2		0.49		
Colorimeter Hunter L		11.88**			
	45		18.52**	26.28**	3.05*
	37.8		259.23**	27.11	171.51**
	29.4		43.39**	1.50	80.32**
	22.2		18.32**	4.44	23.96**
Hunter a		3.78**			
	45		19.16**	1.70	34.21**
	37.8		11.88**	22.58**	0.46
	29.4		11.07**	0.12	24.74**
	22.2		0.66		
Hunter b		5.67**			
	45		2.16		
	37.8		86.11**	6.25*	120.69**
	29.4		7.02*	1.69	11.38**
	22.2		3.20		

[a] A general linear test was used to determine if the quadratic equations calculated from the 1979 and 1980 data were similar. If found to be significant, then for each temperature, the equality of the lines relating the analysis to time were tested. If this was significant, then the quality of the slopes and means were tested.
** Significantly different at 0.01 level.
* Significantly different at 0.05 level.

the sensory method of choice when establishing product deterioration information. This study thereby agrees with Dethmers (1979) and Stone et al. (1974) in that TPP's provide sound information concerning the sensory changes during product deterioration.

Results from the present study also indicated that the tomato paste became unacceptable at a much lower absorbance level than that reported by Hernandez and Feaster (1960). They claimed that absorbance readings above 0.70 in tomato serum was objectionable from a flavor standpoint. The unacceptable absorbance level in the current study, predicted by using a FBM PAT of 3.5 as the point in time when the product became unacceptable, was 0.40 or above.

Prediction of shelf-life

Labuza (1982) stated that the shelf-life of a product can be established with the following information: a measure of the food's deterioration, the level of measurement which represents the end of the shelf-life, the order of the reaction and a measure of the deterioration of the product at least at two temperatures. The measure of the deterioration in this current study was the TPP FBM score and the end of an acceptable shelf-life was a TPP FBM of 3.5. The Office of Technology Assessment (OTA, 1979) stated that the Maillard reaction, which follows zero order reactions kinetics, was found to be the major mode of deterioration in canned fruit and vegetables and that it leads to bitter off-notes. Both Luh et al. (1958) and the current study found an increase in HMF content in tomato paste as the product deteriorated. Furthermore, the present study found the development and increase of bitter flavor intensity as the product deteriorated. The linearity of almost all the changes which occurred in tomato paste determined in this study (Fig. 1-5) indicate that the deterioration of the tomato paste follows zero order kinetics. Four temperatures were examined in the present study to measure the deterioration.

Table 2—Correlation of sensory with instrumental analysis in tomato paste

Sensory analyses	Instrumental analyses	Correlation coefficient[a] 1979 Study	1980 Study	1979 vs 1980[b] To test for equality of Lines	Slopes	Means
Colors PAT	Serum Color	0.928	0.913	2.50	–	–
Aroma PAT	Serum Color	0.777	0.853	5.63**	10.67**	0.49
FBM PAT	Serum Color	0.812	0.872	3.48*	6.21*	0.69
Color PAT	HMF Content	0.926	0.859	14.90*	22.30**	5.41*
Aroma PAT	HMF Content	0.764	0.778	5.67**	1.24	10.05**
FBM PAT	HMF Content	0.769	0.625	7.23**	3.43	10.56**
Color PAT	Hunter L	−0.928	−0.922	30.76**	0.47	61.71**
Aroma PAT	Hunter L	−0.758	−0.883	9.75**	4.64*	13.53**
FBM PAT	Hunter L	−0.742	−0.893	7.37**	1.91	12.61**
Color PAT	Hunter a	−0.894	−0.864	16.31**	0.61	32.25**
Aroma PAT	Hunter a	−0.694	−0.800	13.71**	2.57	24.11**
FMB PAT	Hunter a	−0.738	−0.817	15.00**	0.64	29.58**
Color PAT	Hunter b	−0.825	−0.907	8.91**	0.67	17.27**
Aroma PAT	Hunter b	−0.699	−0.844	4.69*	6.51*	2.59
FBM PAT	Hunter b	−0.670	−0.868	3.38*	4.31*	2.31
Hedonic	Serum Color	−0.814				
Hedonic	HMF Content	−0.773				
Hedonic	Hunter L	0.764				
Hedonic	Hunter a	0.859				
Hedonic	Hunter b	0.686				
Hedonic	Color PAT	−0.796				
Hedonic	Aroma PAT	−0.661				
Hedonic	FBM PAT	−0.686				

[a] Correlation coefficients (r) were significantly different from 0, $P < 0.01$ (Dixon and Massey, 1968); n = 31 for 1979 and n = 32 for 1980.
[b] A general linear test was used to determine if the regressions lines calculated from the 1979 and 1980 data were similar. If the equality of the lines were found to be significantly different, the equality of the slopes and means were tested.
** Significant difference at 0.01 level.
* Significant difference at 0.05 level.

Labuza and Riboh (1982) indicated that the temperature dependence of the deterioration of a food follows the Arrhenius relationship. The deteriorative reaction sensitivity to temperature changes is expressed as the activation energy (E_a) which is derived from the Arrhenius relationship. The E_a's calculated, by using the slope of the Arrhenius plot, from the 1979 and 1980 tomato paste studies were 18 and 23 Kcal/mole, respectively. Labuza (1982) has also expressed the deteriorative reaction sensitivity to temperature as the Q_{10} of the product. The Q_{10} of the product was predicted from the plot of the shelf-life at various temperatures. The shelf-life semi-log plots for both the 1979 and 1980 studies are shown in Fig. 6. According to the 1979 study, tomato paste has a Q_{10} of 3.0 and a shelf-life of about 21 months at 25°C. The 1980 study indicated that the Q_{10} for tomato paste was 3.8 with a shelf-life of about 38 months at 25°C. These findings agree with the Office of Technology Assessment (OTA, 1979) in that canned fruit and vegetables have a shelf-life of 1-3 yr, but disagree with the statement that they have a Q_{10} of 1.5-2.5. This discrepancy may be attributed to the fact that the OTA was making general statements for groups of products and this investigation was product specific.

The 1980 study most likely provided a more reliable indication of shelf-life due to two factors: one, the problem with the 37.8°C chamber in the 1979 study, and two, there may have been a training effect on the TPP. This training effect implies that by the time the 1980 study started, the TPP had more experience tasting tomato products and working as a group.

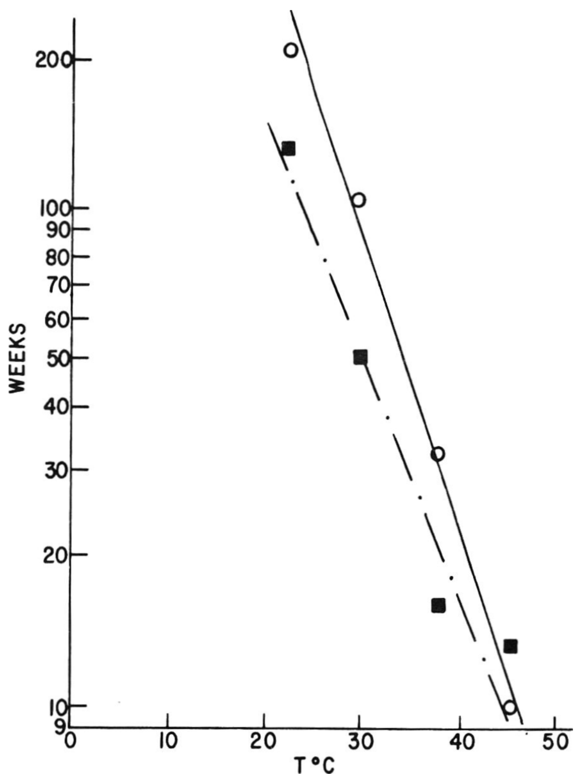

Fig. 6—Time to failure (flavor-by-mouth production acceptability score of 3.5) for tomato paste: 1979 ■—·—■ and 1980 ○——○.

CONCLUSION

ALL THE ANALYSES examined exhibited a change due to the effects of time and temperature; however, the hedonic flavor evaluation and the colorimeter values were not as reliable as the other methods of analysis in describing differences in the rate of the product's deterioration at the temperatures examined. All three instrumental analyses indicated that the product became browner or darker as the product deteriorated. The flavor notes which developed as the product deteriorated were hay, caramelized, burnt, prune and bitter; some of these notes might be attributed to the Maillard browning reaction. The highest correlations between sensory and instrumental testing methods were found between TPP color PAT and any instrumental testing method. The TPP FBM PAT had the highest correlation coefficient for all flavor scores in describing the rate of product deterioration and was used to predict the shelf-life of the product. The instrumental testing method that had the highest correlation coefficient with the FBM score was the serum color. Serum color analysis can now be employed as an inexpensive method of determining product acceptability. The Q_{10} of the product was a 3.8 with a shelf-life of 38 months at 25°C as determined by the 1980 study.

Further studies should be conducted to determine the initial variability in the serum color of the tomato paste. The actual environmental conditions to which the tomato paste is actually subjected to during storage and distribution should also be measured to enable the prediction of the extent of shelf-life used prior to reaching the consumer.

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A Research Note

Effect of Selected Yogurt Cultures on the Concentration of Orotic Acid, Uric Acid and A Hydroxymethylglutaric-Like Compound in Milk After Fermentation

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ABSTRACT

Eight yogurts, each fermented by different strains of *Lactobacillus bulgaricus* and *Streptococcus thermophilus*, were produced, then analyzed using high-performance liquid chromatography (HPLC) for the presence of orotic, uric and 3-hydroxy-3-methylglutaric (HMG) acids. In all yogurts orotic acid decreased 15-53% after fermentation while uric acid levels did not change. A compound was found in all yogurt extracts that co-chromatographed with HMG on HPLC and had a similar UV spectrum to HMG after both were collected from a reverse phase HPLC column. However, the HMG co-elutant did not correspond to HMG on thin-layer or paper chromatography. These results suggest that HMG is not present in milk or yogurt. Fermentation of milk by yogurt strains reduced the concentration of the HMG co-elutant in six of the yogurts, but increased it in two yogurts.

INTRODUCTION

MANN AND SPOERRY (1974) observed that feeding Masai warriors large amounts of fermented whole milk lowered serum cholesterol. Since then others have reported that consumption of large quantities of dairy products, both fermented and nonfermented, lowered human serum cholesterol (Mann, 1977; Marks and Howard, 1977; Hepner et al., 1979). Mann (1977) hypothesized that dairy products contain a cholesterol-lowering "milk factor." Ward et al. (1982) identified uric acid, a purine, as an inhibitor of cholesterolgenesis in human milk. In bovine milk, two pyrimidines, uracil and orotic acid, were identified as inhibitors of cholesterol synthesis (Ahmed et al., 1979; Papa et al., 1980). In addition, 3-hydroxy-3-methylglutarate (HMG) has been proposed as a possible "milk factor" (Mann, 1977), but has not been demonstrated to be present in milk products. This study was designed to test the hypothesis that fermentation of milk by yogurt strains alters the concentrations of orotic acid, uric acid and HMG.

MATERIALS & METHODS

Yogurt culture strains

Two commercial freeze-dried yogurt cultures (CH-1 and CH-2) were obtained from CHR Hansen's Laboratory, Inc. (Milwaukee, WI). These cultures were a 1:1 ratio of *Lactobacillus bulgaricus* and *Streptococcus thermophilus*. The three patented strains of *L. bulgaricus* (201, 202, 203) and two patented strains of *S. thermophilus* (EBC, MC) were provided by Dr. K.M. Shahani (Univ. of Nebraska, Lincoln, NE).

Yogurt preparation

Yogurt mix, containing 13.5% total milk solids, was prepared from fresh skim milk and non-fat dried milk solids (NFDM). The mix was heated to 50°C, homogenized with a hand homogenizer, heated to 90°C and held for 5 min. The heated mix was cooled to 43°C in tap water and sub-divided into 20 mL portions. Active yogurt culture was added (2% v/v) to the mix, stirred, covered with aluminum foil and incubated at 43°C until a pH of 4.6–4.8 was attained (5-6.5 hr). At this point the yogurts were placed in a 4°C cooler and held no more than 1 wk until analysis. Four batches of yogurt were prepared using each strain.

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Yogurt extract preparation

Five grams of yogurt were weighed into a centrifuge tube, then 0.5 mL of 5% (w/v) oxalic acid and 10 mL of 95% ethanol were added. The mixture was agitated for 1 min prior to centrifuging (7000 x *g* for 5 min) at room temperature. The supernatant was filtered through Whatman #4 paper, evaporated in a rotary evaporator, the volumes adjusted to 10 mL with distilled water, again filtered through Whatman #4 paper and finally through a 0.45 μm cellulose acetate filter (Millipore Filter, Bedford, MA) prior to high-performance liquid chromatography (HPLC) analysis. Each extract was analyzed in duplicate.

HPLC analysis

HPLC analysis was conducted using a Waters Associates Chromatography pump (Model 6000A solvent delivery system, Waters Associates, Inc., Milford, MA) with a Waters Intelligent Sample Processor automatic injection module. A Perkin-Elmer (Norwalk, CT) LC-75 Autocontrol variable wavelength detector was used at 208 nm for HMG, and 278 nm for orotic and uric acids, respectively. Eluates were visualized using a Hoveton Instruments (Austin, TX) Omniscribe strip chart recorder. Peak area and retention times were integrated with a Spectra-Physics (Santa Clara, CA) Autolab Minigrator. The column used for separation was a Bio-Rad Laboratories (Richmond, CA) Aminex HPX-87 ion exclusion column (300 mm x 7.9 mm). The solvent was 0.003N H_2SO_4 at a flow rate of 0.6 mL/min.

Qualitative analyses of HMG, orotic and uric acids were conducted by comparing retention time of the unknowns to those of known standards prepared from high purity chemical standards (Sigma Chemical Co., St. Louis, MO). Orotic and uric acids were placed in distilled water and 0.1N NaOH was added to adjust the pH to 8. Heat was then applied until the acid crystals were dissolved. The solutions were cooled to room temperature and made up to volume. Recovery of added HMG, orotic and uric acids were 87%, 82% and 71% respectively. Fractions suspected as HMG were collected from the HPLC waste line, air evaporated to dryness, and volume adjusted to 2 mL with distilled water. Sulfuric acid was removed from this solution by HPLC using a Zorbax (DuPont Instruments, Wilmington, DE) reverse phase octadecyl silane (ODS) column (250 mm x 4.6 mm). The mobile phase was 0.002N HCl at a flow rate of 2 mL/min. Injection volumes were 0.25 to 1 mL and detection was at 208 nm. The collected fractions were evaporated almost to dryness using a rotary evaporator (water bath at 30°C max) and diluted to 2 mL with distilled water. Standards of known HMG were prepared in a similar manner.

Thin layer and paper chromatography

The thin-layer chromatography procedure was based on the procedure of Stahl (1965). Pre-poured plates (20 x 20 cm) of silica gel G (Supelco, Inc., Bellafonte, PA) were utilized. The solvent mixture consisted of 95% ethanol, water and 25% ammonium hydroxide (100:12:16). Known HMG was applied to the plates in 20-100 μL volumes.

Paper chromatography was based on a procedure by Amerine (1971). HMG and the suspect HMG co-elutant were spotted on a 20 x 20 cm sheet of Whatman #1 filter paper at levels of 60 μL (HMG) to 240 μL (HMG co-elutant). Development was in cylindrical glass chromatography chamber using n-butanol, 90% formic acid, water (9.2:1:9.2) using the top layer of the two immiscible layers as the solvent by an ascending mode.

Table 1—Concentration of orotic acid, uric acid and a hydroxymethylglutaric acid co-elutant in unfermented yogurt mix and yogurts (concentration, ppm)

Culture strain	Orotic acid		Uric acid		Hydroxymethylglutaric acid Co-elutant[b]	
	Mix	Yogurt	Mix	Yogurt	Mix	Yogurt
EBC/201	38.0 ± 0.5	25.6[a] ± 0.9	14.6 ± 0	14.7 ± 1.6	300 ± 14	128 ± 7
EBC/202	38.0 ± 0.5	25.7[a] ± 1.4	14.6 ± 0	14.6 ± 0.1	300 ± 14	129 ± 14
EBC/203	38.0 ± 0.5	32.4[a] ± 0.2	14.6 ± 0	15.0 ± 0.2	300 ± 14	113 ± 149
MC/201	38.0 ± 0.5	20.5[a] ± 0.7	14.6 ± 0	14.8 ± 0	300 ± 14	161 ± 6
MC/202	38.0 ± 0.5	23.6[a] ± 1.4	14.6 ± 0	15.4 ± 1.4	300 ± 14	221 ± 73
MC/203	38.0 ± 0.5	32.3[a] ± 0.9	14.6 ± 0	14.2 ± 0	300 ± 14	216 ± 30
CH-1	40.5 ± 3.5	23.2[a] ± 1.3	17.2 ± 0.1	16.8 ± 0.4	139 ± 124	180 ± 7
CH-2	36.5 ± 6	17.3[a] ± 1.2	14.0 ± 1.8	15.2 ± 0.6	94 ± 7	156 ± 53

[a] Significantly different from unfermented mix ($p < 0.05$).
[b] Calculated using absorption values for HMG.

A Beckman UV-Visible Model 35 Spectrophotometer (Beckman Instruments Inc., South Pasadena, CA) was used to determine the ultraviolet (UV) spectra of standards and the HMG co-elutant compound.

RESULTS & DISCUSSION

OROTIC ACID CONCENTRATION decreased significantly ($P < 0.05$) during fermentation (Table 1). This finding is similar to the observations of Marsili et al. (1981), Alm (1982) and Okonkwo and Kinsella (1969). The concentrations of orotic acid found in the yogurts in this study ranged from 17.3–32.4 ppm, which are less than the 72–83 ppm in milk and 34–72 ppm in yogurt reported by Marsili et al. (1981), Richardson (1978), Larson and Hegarty (1979) and Okonkwo and Kinsella (1969).

Unlike orotic acid, uric acid did not change significantly during fermentation (Table 1). The concentrations of uric acid found in the unfermented yogurt mixes are in close agreement with the 20–22 ppm reported in milk (Shahani and Sommer, 1951; Marsili et al., 1981). The concentration of uric acid found in all the yogurts analyzed (14.2–18.2 ppm) was less than the 31 ppm reported by Marsili et al. (1981).

Hydroxymethylglutarate was not found in milk or yogurt. Retention time and ultraviolet spectra comparisons of known HMG and the HMG co-elutant were very similar. The HMG co-elutant peak consistantly eluted within ±12 sec of HMG. However, when the HMG co-elutant peak was collected from the HPLC waste line, concentrated, then rechromatographed on the ODS column to remove the concentrated, then rechromatographed on the ODS column to remove the concentrated sulfuric acid, only one of the two resulting compounds had the same retention time as known HMG subjected to the same treatment. When further identification of the HMG co-elutant peak was attempted using paper and thin-layer chromatography, no spot with an Rf corresponding to identically treated HMG was detected.

There were no significant differences in yogurt strains in their effects on orotic and uric acid concentrations. However, while fermentation by six of the strains decreased the concentration of the HMG co-elutant, two strains, CH-1 and CH-2, may have produced this compound, since its concentration was greater in the fermented product. There appears to be strain differences in the production of the HMG co-elutant, but not orotic or uric acids.

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A Research Note

Screening of Milk and Milk Products for Thermonuclease

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ABSTRACT

Out of 208 samples of dairy products, 17.8% contained thermonuclease. Staphylococcal TNase (6-49 μg/100 mL or g) was detected in 14.4% samples that also contained TNase positive *S. aureus* and their enterotoxins. Enterotoxin A was recorded most frequently. Incidence of TNase was maximum (30.4%) in raw cow's milk followed by nonfat dry cow's milk (25.9%) and Kulfi (25.0%). The most frequent contaminants in the seven nonstaphylococcal TNase positive samples (6 – 16 μg per 100 mL org) were TNase-positive enterococci. TNase-positive *B. cereus* was recovered from a TNase-positive sweetened condensed milk sample. Unidentifiable TNase producing molds were detected in three TNase-positive samples.

INTRODUCTION

THE PRODUCTION of thermonuclease (TNase) by a majority of enterotoxigenic strains of *Staphylococcus aureus* (Lachica et al. 1969; Lachica et al., 1971a, b; Barry et al., 1973; Raymon et al., 1975; Sperber, 1976) has now been well documented as one of the major criteria in the identification of enterotoxigenic *S. aureus* (Baird-Parker, 1974; Sperber, 1976). The TNase test has also been proposed as a rapid means of screening foods for growth of *S. aureus* and potential enterotoxin production (Lachica et al., 1972; Chesbro and Auborn, 1967; Tatini et al., 1975; Koupel and Deibel, 1978; Park et al., 1980; Batish et al., 1978; Rao et al., 1980). However, in view of occurrence of TNase producing microorganisms other than *S. aureus* in foods (Thomas and Nambudripad, 1974; Park et al., 1980; Bissonnette et al., 1980; Batish et al., 1982), the validity of the TNase test for determining the likely presence of staphylococcal enterotoxins in the foods can be subjected to criticism. Hence in the present investigation, an attempt has been made to screen a large number of samples of milk and milk products for the presence of thermonucleases of staphylococcal as well as nonstaphylococcal origin.

MATERIALS & METHODS

A TOTAL of 208 samples of milk and milk products were procured from local markets, commercial dairies and the Experimental Dairy and Cattle Yard of N.D.R.I., Karnal. Thermonuclease was extracted from the test samples by the procedure of Tatini et al. (1976) after suitable modifications (Batish et al., 1982). TNase extracts were prepared from the samples after adjusting their pH to 4.5 as well as at 3.8. The crude extracts of the enzyme following the above procedures were divided into two aliquots. One of the aliquots was boiled for 15 min and the other for 1 hr. The TNase assay system was carried out with toluidine blue DNA agar medium (Lachica et al., 1971a, b) adjusted to pH 6.7 and 9.0 (poured into 95 mm diameter plastic petri plates) to differentiate between staphylococcal and enterococcal TNase. Staphylococcal TNases are most active at pH 9.0 and enterococcal TNase acrivity is best exhibited at pH 6.7 (Batish et al., 1982). After solidification, agar plates were stored at 5 – 7°C until further use. Two mm wells were made in each plate and 5 μL TNase extracts were gently transferred to each well. The plates were incubated for 4 hr at 37°C. Development of pink zones around the wells was a positive indication of the presence of thermonuclease. A standard curve was prepared using purified micrococcal nuclease (Sigma) in tris-buffer (pH 8.5) to determine the quantity of thermonuclease in each sample.

Enterotoxins of *Staphylococcus aureus* were extracted from the thermonuclease-positive samples (Casman, 1967) Microslide gel double diffusion tests (Casman et al., 1969) were carried out to detect enterotoxin in the extract using standard enterotoxin antisera supplied by Dr. M. S. Bergdoll, Food Research Institute, Univ. of Wisconsin (Madison, WI). Thermonuclease-positive samples that did not show the presence of staphylococcal enterotoxins were examined for the presence of thermonuclease-positive *S. aureus*, enterococci and Bacillus species using Baird-Parker's agar (ICMSF, 1978), citrate azide agar (Saraswat et al., 1963), and tryptone dextrose agar (APHA, 1972), respectively. A total of 20 colonies each of the isolates growing on the above media were randomly selected, isolated and tested for TNase production after growth in BHI and 10% reconstituted nonfat dry cow's milk for 24 hr at 37°C. The TNase positive enterococcal isolates were characterized and identified on the basis of their morphological, biochemical and physiological characteristics using the procedure of Facklam (1974) and Diebel and Seeley (1974). Bacillus species were identified as per the scheme presented by Gibson and Gordon (1974).

RESULTS & DISCUSSION

THE INCIDENCE of samples of milk and milk products positive for thermonuclease of staphylococcal and nonstaphylococcal origin is shown in Table 1. Although 37 samples showed the presence of thermonuclease, staphylococcal TNase was detected in only 30 samples of the 208 samples analyzed. This was confirmed by the presence of TNase-positive *S. aureus* as well as staphylococcal enterotoxins in the samples. The maximum incidence of thermonuclease was observed in seven samples of raw cow's milk followed by seven nonfat dry cow's milk and three infant foods. The maximum concentration of TNase occurred in a sample of raw cow's milk. There was a preponderence of enterotoxin A in TNase positive samples. Our results are consistent with the earlier findings of Batish et al. (1978), Rao et al. (1980), and Singh et al. (1980) who also detected thermonuclease in samples of milk and milk products. Staphylococcal enterotoxins were also detected by the above workers in the thermonuclease positive samples, thereby indicating the usefulness of TNase test in screening foods for the presences of enterotoxins. Similar findings were also observed by Tatini et al. (1975) in cheese buffer, nonfat dry cow's milk and dried malted milk samples. The high incidence of thermonuclease in milk and milk products examined in the present investigation suggests that milk and milk products were handled under nonhygienic conditions.

Distribution of nonstaphylococcal TNase-positive samples contaminated with the predominating microorganisms capable of TNase production is presented in Table 2. The concentration of TNase in these samples ranged from 6 μg/100 mL (ice-cream) to 16 μg/100 mL in raw cow's milk. All seven samples contained bacteria other than *S. aureus* as major contaminants. The major contaminating organisms belonged to 'Enterococcus' group and Bacillus species. TNase-positive enterococci were recovered from a sample each of raw cow's milk, sweet cheese, cheddar

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Table 1—Incidence of samples of milk and milk products positive for thermonuclease[a]

Type of samples	No. of samples tested	No. of samples positive for thermonuclease	No. of positive samples showing *S. aureus*	Level of staphylococcal TNase µg/100 mL org.	Type of staphylococcal enterotoxin
Raw cow's milk	23	7 (30.4)	6 (26.7)	19–49	A, C, D
Pasteurized cow's milk	17	1 (6.9)	1 (5.9)	12	A
Sweet cheese	10	2 (20.0)	1 (10.0)	26	A
Cheddar cheese	25	5 (20.0)	4 (16.0)	9–31	A, B
Processed cheese	10	0	0	–	–
Butter	20	2 (10.0)	2 (10.0)	8–12	A
Kulfi-mix	20	5 (25.0)	3 (15.0)	16–32	A, B, C
Kulfi-mix	20	2 (10.0)	2 (10.0)	29	A, B
Ice-cream	10	2 (10.0)	2 (10.0)	29	A, B
Sweetened condensed milk	10	1 (10.0)	0 (10.0)	–	–
Nonfat dry cow's milk	27	7 (25.0)	7 (25.9)	7–36	A, B, C
Infant food (spray-dried)	16	3 (18.7)	3 (18.7)	6–13	A, B
Total:	200	37 (17.8)	30 (14.4)		

[a] Data in parentheses indicate percentage; –, not detected.

Table 2—Distribution of TNase-positive samples of milk and milk products by organisms other than Staphylococci

Type of samples	No. of samples	Amount of TNase (µg/100 mL or g)	Predominant contaminants Enterococcus species[a]	TNase positive Bacillus species	TNase positive Molds[b]
Raw cow's milk	1	16	+(S.f.f.)	–	–
Sweet cheese	1	9	+(S.f.z.)	–	–
Cheddar cheese	4	11	+(S.f.z.)	–	–
Kulfi	2	i) 13	+(S. fae)	–	+
		ii) 14	+(S. fae)	–	+
ice cream	1	6	+(S. fae)	–	+
Sweetened condensed milk	1	8	–	+(B.C)	–

[a] Parentheses used for abbreviation of bacteria species names: S.f.f. = S. faecalis var. faecalis; S.f.z. = S. faecalis var. zymogenes; S. fae = S. faecium; B.c. = Bacillus cereus.
[b] Unidentified molds; +, present; —, absent.

cheese and ice cream and two samples of Kulfi. They were identified as *S. faecalis* var. *faecalis; S. faecalis* var. *zymogenes* (two samples); *S. faecium.* TNase positive Bacillus isolates recovered from a sweetened condensed milk sample were identified as *B. cereus.* In addition, a few mold isolates capable of TNase production were isolated from two Kulfi and one ice-cream samples, but they could not be identified. This corroborates the findings of Bissonnette and coworkers (1980) who showed thermonuclease production by *Bacillus* sp. and enterococci in naturally contaminated cheese. TNase production, in addition to *S. aureus*, was demonstrated by some strains of Bacillus sp. and a few strains of group D streptococci (Park et al., 1980). In the present investigation TNase of *S. aureus* was differentiated from that of Bacillus sp. and enterococci on the basis of extraction of Staphylococcal TNase at low pH 3.8 as suggested by Bissonnette et al. (1980), and greater heat resistance of TNase as suggested by Park et al. (1980). By using both these steps, the interference of nonstaphylococcal TNase could be eliminated to a greater extent, since only TNase of *S. aureus* was able to resist these treatments. In our earlier study (Batish et al., 1982), TNases of enterococci were found to be active both at slightly acidic pH 6.7 as well as alkaline pH 9.0 whereas TNases of *S. aureus* could not be clearly demonstrated at pH 6.7. Hence, it can be concluded that if a correct interpretation of contamination of dairy products by *S. aureus* is to be made, TNase extraction must be followed at low pH, i.e. 3.8, and the extract should be boiled for 1 hr before carrying out the TNase test for determining the likely presence of enterotoxin in the food sample. TNase assay should be conducted at pH 6.7 as well as pH 9.0 in order to further differentiate between staphylococcal TNase and enterococcal TNase.

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A Research Note

Use of *Candida tropicalis* ATCC 9968 to Adjust the pH of a Natural Lactic Acid Fermentation of Cornmeal

M. E. BASELER and M. L. FIELDS

ABSTRACT

After 3 days of a natural lactic acid fermentation of whole kernel cornmeal, the pH was 3.78 with a titratable acidity of 0.91% at 32°C (1:4 w/v solids to water). These solids were diluted (1:12 w/v), autocalved at 121°C for 15 min and fermented with *C. tropicalis* for 7 days at 32°C, bringing the pH to 6.5 and 0.03% acidity. After both fermentations, the % relative nutritive value increased significantly (from 74.09% to 81.22%) and so did riboflavin (from 0.22 to 0.56 mg/100g). Both thiamin and niacin decreased significantly (0.42 to 0.20 mg/100g and 2.13 to 1.94 mg/100g, respectively).

INTRODUCTION

IN PREVIOUS RESEARCH, we found that indigenous microflora occurring on legumes (Zamora and Fields, 1979a, b) produced improvement in riboflavin, methionine, isoleucine and tryptophan contents and percent relative nutritive value (a measure of the amino acid balance). In addition, raffinose and stachyose decreased. A natural fermentation of cereals (Hamad and Fields, 1979a; Au and Fields, 1981; Tongnual and Fields, 1979; Kazanas and Fields, 1981; Lay and Fields, 1981; Umoh and Fields, 1981; Tongnual et al., 1981; Wang and Fields, 1978) also produced nutritional improvements due to a natural fermentation. The fermentation was a lactic acid fermentation (Zamora and Fields, 1979a, b; Fields et al., 1981).

In foods made from these fermented materials, the pH was adjusted to their original pH or to a more alkaline pH with baking soda (Zamora and Fields, 1979c; Hamad and Fields, 1979b). Sensory panelists in evaluating a chickpea soup with an unadjusted pH (pH 4.2) reported that the soup had an acid flavor which they found disagreeable (Zamora and Fields, 1979c). Because of the necessity of adding baking soda, which can also influence the flavor if added in large quantities (Charley, 1970), this research was instituted to determine the influence of using *Candida tropicalis* to raise the pH but still retain the nutritional benefits derived from the naturally occurring lactic acid fermentation.

MATERIALS & METHODS

Sample preparation

Five bags of corn purchased in Columbia, MO were cleaned, washed, dried, and ground through a 1-mm screen in a Wiley mill. Tap water (2:4 w/v) was added to 150g ground corn and incubated at 32°C for 3 days in replicated 2000-mL Erlenmeyer flasks covered with aluminum foil. The pH, titratable acidity as lactic (% TA) and reducing sugars (Bernfeld, 1955) were determined daily for the entire experiment.

After 3 days, tap water (1:12 w/v) was added to the fermented sample and the flask was stoppered with gauze-wrapped cotton. The mixture was autoclaved for 15 min at 121°C. *Candida tropicalis* ATCC 9968 was selected for use because it can metabolize pentoses and soluble starch and because it had been used previously for yeast protein (ATCC, 1982). Yeast (*Candida tropicalis* ATCC 9968) was added to four replicate flasks per treatment. The inoculum was 1 mL of a suspension of a 2-day old cutlure of *C. tropicalis*, ATCC 9968 grown in yeast malt broth, centrifuged, and suspended in sterile distilled water to give 50% transmittance at 600 nm in a Bausch and Lomb spectrophotometer. When the pH of the corn rose to 6.5, the sample was dried for 24–48 hr at 50–70°C.

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Analytical procedures

Moisture and nitrogen were determined according to AOAC (1975). Stott and Smith's method (1963) was used to determine % relative nutritive value (% RNV). Sample preparation for niacin and riboflavin determinations followed the procedure of Freed (1966). Preparation of samples for thiamin followed the Sarett and Cheldelin's (1944) procedure. Preparation of working standards, stock cultures, inocula and assay tubes for all vitamins were according to the procedures of Difco (1977).

Statistical analyses

An analysis of variance (Snedecor and Cochran, 1980) was used to determine the significance of the data obtained from four replications of vitamin and three replications of % RNV assays. Duncan's (1955) multiple range test was used to locate significant differences between the means of the fermented samples and nonfermented control.

RESULTS & DISCUSSION

THE CHARACTERISTICS of a natural lactic acid type of fermentation (lowering of pH and increased titratable acidity) during the first 3 days and the fermentation with *C. tropicalis* ATCC 9968 are shown in Table 1. During the first day of the natural lactic acid fermentation, the level of reducing sugars increased 4.38 times but then decreased on the second and third days. The increase and decrease can be attributed to action of the microflora during the fermentation.

During autoclaving, the quantity of reducing sugars or reducing substances increased 3.8 times due to the auto-

Table 1—Means of pH, titratable acidity and reducing sugars after fermenting cornmeals by two methods

Type of fermentation	Days	pH	% acidity	Reducing sugars (mg glucose/mL sample)
Natural lactic acid[a]	0	6.38	0.06[b]	1.34
	1	4.38	0.50	5.88
	2	3.92	0.76	2.40
	3	3.78	0.91	2.28
Candida tropicalis ATCC 9968[c]	0	3.98[d]	0.31	8.64[e]
	1	3.91	0.31	8.42
	2	3.95	0.30	8.74
	3	3.92	0.28	6.99
	4	3.98	0.26	6.73
	5	4.30	0.19	5.69
	6	5.26	0.10	4.94
	7	6.65	0.03	5.88

[a] N=4. Ratio of cornmeal solids to tap water 1:4 (w/v) at 32°C.
[b] Expressed as lactic acid.
[c] Fermentation after 1:12 (w/v) and autoclaving 121°C for 15 min. Fermentation by C. tropicalis ATCC 9968 followed the natural lactic acid fermentation.
[d] pH taken immediately after autoclaving.
[e] Reducing sugar content immediately after autoclaving.

Table 2—Relative nutritive value (% RNV),[a] thiamin,[b] niacin,[b] and riboflavin[b] contents of fermented and nonfermented cornmeal

Nutrient evaluation	Fermented[c]	Non-fermented[c]
% RNV (protein quality)	81.22[x]	74.09[y]
Thiamin mg/100 g	0.20[x]	0.42[y]
Niacin mg/100 g	1.94[x]	2.13[y]
Riboflavin mg/100 g	0.56[x]	0.22[y]

[a] N=3.
[b] N=4. Values calculated on dry basis.
[c] Means with different letters (x or y) are significantly different at the 5% level.

claving temperature and the acidity of the fermented cornmeal. *C. tropicalis* can assimilate DL-lactic acid (Uden and Buckley, 1971) so it was expected that as time proceeded, the pH increased and the % acidity decreased. However, at the same time the level of reducing sugars decreased. The utilization of acid in preference to reducing sugars is not easily explained except that the test for reducing sugars was actually measuring mainly dextrins. Miller (1982) stated that fermentation of polysaccharides, such as starch, was done by relatively few yeasts, and then generally at a reduced rate. Desrosier and Desrosier (1977) stated that in fermentations, sugars were attacked before alcohols, and alochols before acids. In our case, starch was not fermented by our strain of *C. tropicalis*, but some strains do assimilate soluble starch (amylodextrin). Presumably, the yeast was metabolizing the lactic acid as well as other reducing substances (not necessarily sugars).

C. tropicalis did not induce a rather large change in pH until the fifth day. Since only 1 mL of inoculum was used, a population build up was needed to bring about a change of this magnitude. Larger inocula might decrease the time to bring about a decrease in pH. Further research dealing with inoculum volume is indicated.

Data in Table 2 indicate that, in addition to the increase in pH, the process produced an increase in the % RNV (amino acid balance) and the increase of riboflavin. These increases were due to both the natural lactic acid fermentation and the *C. tropicalis* fermentation. Both lactic acid producing bacteria and some *Candida* yeasts synthesize this vitamin (Wagner-Jauregg, 1972).

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A Research Note

Fermentation of Corn Gluten Meal with *Aspergillus oryzae* and *Rhizopus oligosporus*

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ABSTRACT

PER values determined for corn gluten meal (CGM) and CGM fermented with *Aspergillus oryzae* NRRL 1988 were not significantly different ($P > 0.05$) and both diets failed to meet maintenance requirements of rats. In order to characterize some of the changes that occur during fungal fermentation, CGM was also fermented for 4 days at 28°C with *A. oryzae* NRRL 1988 and NRRL 506 and *Rhizopus oligosporus* NRRL 2710 and NRRL 2549, respectively. Proteolytic activity, pH, and nitrogen content increased rapidly between 20 and 70 hr for all the fungi. Decreases in some amino acids were observed, possibly due to their catabolism by the molds. Lysine as a proportion of total essential amino acids released by pepsin and pancreatin *in vitro* was increased as a result of these fungal fermentations.

INTRODUCTION

CORN GLUTEN MEAL (CGM) is the 60% protein coproduct obtained during the wet-milling of corn. The protein is very low in lysine and tryptophan (Wall and Paulis, 1978) and consequently is of poor nutritional quality. Fermentation with proteolytic fungi has been shown to alter the composition and, in some cases, improve the nutritional quality of protein in various substrates. Fungal fermentation of peanuts and peanut flour has been shown to alter their amino acid composition (Quinn et al., 1975; Cherry et al., 1976; Cherry and Beuchat, 1976). Changes in protein nutritional quality, however, were not observed or were not determined. Soybeans fermented with *Aspergillus oryzae* NRRL 506 showed increases in lysine which were thought to have contributed to improved weight gain and feed efficiency of broiler chickens (Chah et al., 1976). Murthy (1978) reported that the protein efficiency ratio (PER) was improved when groundnut/soybean mixtures were fermented with *Rhizopus oligosporus* NRRL 2549. Wang et al. (1968) observed that the PER of wheat and wheat/soybean mixtures were significantly improved by fermentation with *R. oligosporus* NRRL 2710, although that of soybeans alone was unchanged. The improvement in PER could not be explained by changes in amino acid composition and was attributed to proportionally higher levels of lysine and histidine released by digestive enzymes, pepsin and pancreatin, as determined in vitro.

The purpose of the present study was to determine the effect of fermentation of CGM with certain proteolytic fungi on the nutritional quality of the protein. In addition, proteolytic activity, pH, nitrogen content, total amino acid composition and amino acids released by digestive enzymes in vitro, were studied to characterize the changes that occur during fungal fermentation of this substrate.

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MATERIALS & METHODS

CGM from a commercial source was fermented with *A. oryzae* NRRL 1988 and 506 and *R. oligosporus* NRRL 2710 and 2549, respectively. CGM (40.0g) was weighed into 15 x 90 mm Petri dishes and autoclaved for 20 min at 121°C. Spore suspensions were prepared by the addition of 5 mL of sterile pH 7.0, 0.1M phosphate buffer to each of two slants of the desired organism, gentle agitation to liberate spores, and aseptical transfer of both into 700 mL of sterile pH 7.0, 0.1M phosphate buffer. A 25.0 ml portion of the resulting suspension was pipetted into four Petri dishes that were prepared for each day of fermentation. After incubating at 28°C for 0, 1, 2, 3 or 4 days, contents of two of the Petri dishes were assayed immediately for proteolytic activity, while the remaining two were steamed for 5 min to inactivate enzymes, lyophilized, and saved for subsequent analysis.

Proteolytic activity was determined as "hemoglobin units on tyrosine basis" as described in AACC Method 22-62 (1983). The pH of fermented CGM was determined from 1.0g of the lyophilized sample suspended in 25.0 mL distilled, deionized water. Moisture and nitrogen contents were determined by vacuum oven and micro-Kjeldahl methods, respectively (AOAC, 1980). Starch was determined by AACC Method 76-20 (1983).

Amino acid compositions were determined by hydrolyzing samples with 6N HCl under reflux for 24 hr. Methionine and cystine were determined as methionine sulfone and cysteic acid, respectively, as described by Moore (1963). Amino acids were separated on a Dionex D 300 amino acid analyzer. Tryptophan was determined by hydrolyzing samples with 6N NaOH at 110°C for 22 hr in vacuum-sealed tubes, followed by separation on a Beckman 120C amino acid analyzer. Results were normalized to 95% nitrogen recovery.

The substrates were also subjected to pepsin followed by pancreatin digestion as described by Akeson and Stahman (1964). Samples containing 100 mg protein were incubated with 1.5 mg pepsin in 15 mL of 0.1N HCl at 37°C for 3 hr. The digestion mixtures were neutralized with 7.5 mL of 0.2N NaOH and incubated for an additional 24 hr after addition of 4 mg pancreatin in 7.5 mL of pH 8.0 phosphate buffer. The undigested proteins and larger peptides were removed by picric acid. Amino acids were separated on the Dionex D 300 amino acid analyzer.

RESULTS & DISCUSSION

IN ORDER TO GAIN a better assessment of the nutritional quality of CGM protein, a preliminary study was conducted to determine its PER (AOAC, 1980). CGM that had been fermented with *A. oryzae* NRRL 1988 in an enriched substrate reported to enhance its proteolytic activity (Maxwell, 1952) was also included in the preliminary study. Although the organism grew well on CGM and proteolytic activity reached high levels, there was no significant difference ($P > 0.05$) in PER values for CGM and fermented CGM. Both diets failed to meet maintenance requirements of the rats and gave PER values of −0.10 and 0.00 (adjusted for casein = 2.50) for CGM and fermented CGM, respectively.

Fig. 1 shows the development of proteolytic activity, changes in pH, and nitrogen content during fermentation of CGM with the fungi used in the present study. Proteolytic activity and pH leveled off by 72 hr for all of the organisms indicating that they had reached the stationary growth phase. Changes in nitrogen content during fermentation followed a similar pattern except that *R. oligosporus*

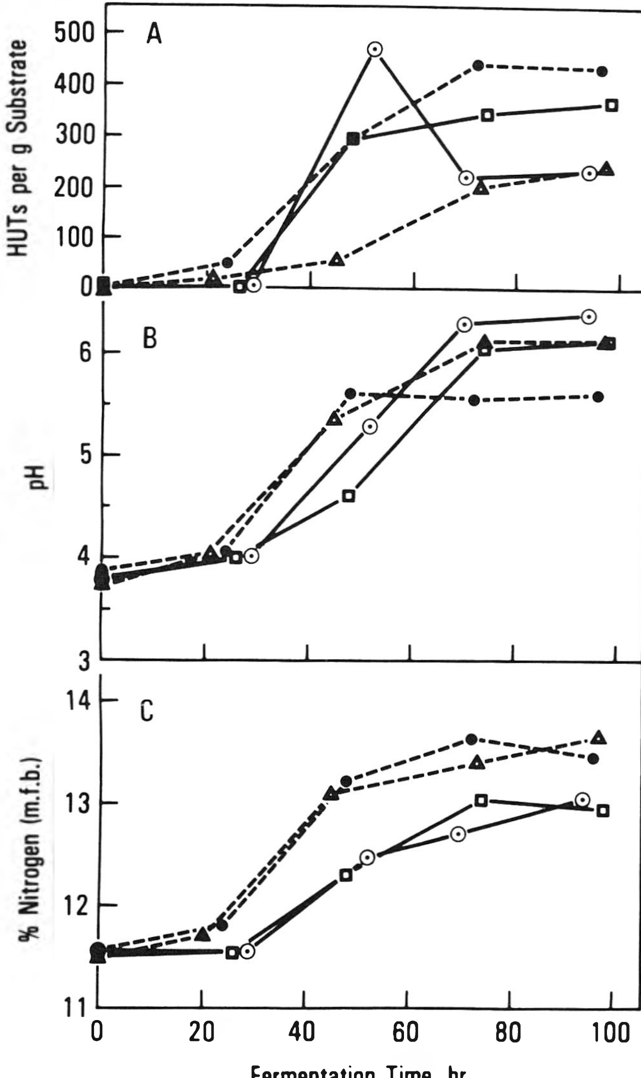

Fig. 1—Changes in (A) proteolytic activity, (B) pH, and (C) nitrogen content of CGM during fermentation: ○ — ○ Aspergillus oryzae NRRL 1988; □ — □ Aspergillus oryzae NRRL 506; △ - - - - △ Rhizopus oligosporus NRRL 2710; ● - - - - ● Rhizopus oligosporus NRRL 2549.

strains increased it at a faster rate and reached higher levels than *A. oryzae* strains. Increases in nitrogen content can be attributed to the loss of non-nitrogenous volatiles resulting from fungal metabolism as explained by Wang et al. (1968) and Quinn et al. (1975). Our increases were greater than those observed by Quinn et al. (1975) on peanut flour using *A. oryzae* 1988 and *R. oligosporus* 2710 at the same fermentation times and temperature. This may be due, in part, to a higher level of fermentable carbohydrate in CGM as compared to peanut flour. CGM contains 12% starch initially, and analysis of CGM fermented by *R. oligosporus* for 72 hr indicated that the starch had been completely utilized.

The levels of most amino acids in the substrates were not affected after 3 days of fermentation, though changes in some were noted and are presented in Table 1. The levels of glutamic acid, proline, leucine, arginine and tryptophan were decreased in varying degree by some of the organisms. Small increases in lysine were observed for three of the molds and increases in ammonia levels of those samples suggested that catabolism of some amino acids may have taken place. Decreases observed in the essential amino acid,

Table 1—Levels of selected amino acids in CGM before fermentation and after 3 days of fermentation with A. oryzae NRRL 1988 and NRRL 506, and R. oligosporus NRRL 2710 and NRRL 2549

	g Amino acid/16g of Nitrogen				
		3 Day Fermented substrates[a]			
		A. oryzae		R. oligosporus	
Amino acid	CGM[b]	1988	506	2710	2549
Glutamic acid	23.10 ± 0.74	22.83	21.60	20.08	23.25
Proline	10.47 ± 0.73	8.31	8.65	7.66	9.63
Leucine	16.19 ± 0.71	14.97	13.81	13.94	15.67
Arginine	4.40 ± 0.42	3.60	4.87	5.11	3.79
Tryptophan	0.40 ± 0.03	0.42	0.35	0.27	0.24
Lysine	2.10 ± 0.23	2.60	2.76	2.82	2.03
Ammonia	2.16 ± 0.22	3.15	2.99	3.17	2.39

[a] Results for each organism are the means of two runs on the amino acid analyzer from a single hydrolysate.
[b] Represents the means of 0 day controls for each organism (4 hydrolysates, 2 runs/hydrolysate) and the standard deviation among the hydrolysates (d.f.=3).

tryptophan, in *R. oligosporus* fermented CGM could have a detrimental effect on the nutritional quality of the protein.

Amino acid analysis of pepsin-pancreatin digests showed that lysine as a proportion of total essential amino acids (EAA) released by digestive enzymes in vitro increased by the end of fermentation (4 days) for all of the fungi tested. *A. oryzae* 1988 was distinguished from the other molds during the first 48 hr of fermentation by differences in the proportions of EAAs released by pepsin and pancreatin. These differences, however, were not apparent later in the fermentation. These results suggest that the proteolytic enzyme system produced by *A. oryzae* 1988 when grown on CGM may differ from that of the other molds. More study is needed, however, to determine how fungal proteolysis affects the release of EAAs by pepsin and pancreatin in vitro and how it relates to the in vivo release of essential amino acids.

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A Research Note

Nutritional Composition of Corn and Flour Tortillas

GUADALUPE SALDANA and HAROLD E. BROWN

ABSTRACT

Corn and flour tortillas were sampled at 2-wk intervals for 6 wk from five tortilla factories and the nutritional composition determined. Corn tortillas were found to be slightly lower in protein, thiamin, riboflavin and niacin when compared to white enriched bread. A lowering of the pH of the corn masa to 7.3 or lower improved the retention of these vitamins. Flour tortillas were found to be comparable in nutrition to white enriched bread when enriched wheat flour was used in the manufacture of tortillas. A wide variation was found to exist in the nutrient composition of both corn and flour tortillas manufactured by the different factories. This variation is attributed to different formulation procedures at the tortilla factories.

INTRODUCTION

THE TORTILLA is a basic food item in the diets of many Americans of Hispanic origin. Tortillas are used in the preparation of such traditional Mexican foods as the enchilada, taco and chalupa and serve as a substitute for bread. The flour tortilla is used primarily as a bread substitute. Some schools, particularly those with high Hispanic enrollment, serve tortillas in lieu of bread in their school lunch programs. Knowledge of nutritional composition of tortillas becomes important to nutritionists for the formulation of well balanced meals in the school lunch programs.

The nutritive content and quality of proteins derived from corn and corn products have been the subject of many research investigations. Cravioto et al. (1945) determined the nutritive value of the Mexican corn tortilla. Losses in thiamin, riboflavin and niacin were relatively small with a 40% loss in carotene from yellow corn tortillas. Significant increases in calcium, phosphorus and iron occurred due to the treatment of the corn with lime water. Cravioto et al. (1952a) found that the nutritive value of corn tortillas was higher in both amount and quality of proteins when compared to whole wheat, whole wheat bread and white bread (French or bolillo bread) commonly used in Mexico. Lysine and tryptophan are known to be deficient in corn (Mitchell and Smuts, 1932) and considerable changes occurred in other amino acids as corn was processed into tortillas (Massieu et al., 1949). Bressani et al. (1958) in a more thorough study, confirmed that the nutrient losses of corn during preparation of tortillas were both of a chemical and physical nature. Bressani and Scrimshaw (1958) showed that the solubility of zein, a poor quality protein fraction in corn, was decreased while the rate of release of most of the essential amino acids was increased as a result of the lime-heat treatment given to the corn kernels. These results explain the findings of Cravioto et al. (1952b) who found that corn tortillas had a better growth promoting value on rats than raw corn. Katz et al. (1974) found that alkali cooking of corn resulted in higher glutelin solubility, higher lysine and niacin contents, and a favorable ratio of isoleucine to leucine.

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Although the alkali heat treatment given to corn enhances the nutritional value of corn tortillas, their nutritional value as a dietary source is still at best marginal. Sure et al. (1953) improved the protein efficiency of corn by adding those amino acids known to be deficient: lysine, tryptophan, and threonine. Racotta et al. (1979), Bressani and Marenco (1963) and McPherson and Ou (1976) obtained similar success by supplementing the corn proteins with protein-rich materials of either animal or vegetable origin.

The objective of this work was to determine the nutritional composition of corn and flour tortillas as supplied to the general public.

MATERIALS & METHODS

SAMPLES consisting of six packages each of corn and flour tortillas were obtained from five local factories. Replicate samples were obtained at 2-wk intervals for 6 wk and on the days of manufacture. A 200g composite of each sample was obtained by coring several times through each stack of tortillas with a 1.27 cm diameter cork borer. A food grinder fitted with a disc having 5 mm openings was used to grind the composite samples. The ground samples were stored at 4.5°C until analyzed.

Prior to analyses, samples were brought into the laboratory and allowed to reach room temperature. The pH of corn tortillas was measured by thoroughly mixing a 25g sample with 25 mL distilled water using a Corning Model 10 pH meter. The moisture (par. 14.002), ash (par. 14.006), fat (ether ext. par. 14.018), crude fiber (par. 14.020), β-carotene (par. 43.014), protein (Kjeldahl, N x 6.25 par. 2.049), thiamin (fluorometric, par. 43.024), riboflavin (fluorometric par. 43.039) and niacin (turbidimetric, par. 43.102) were determined by methods of the AOAC (1975). Vitamin C was determined by the method Nelson and Samens (1945). Calcium, iron, potassium and sodium were determined with a Perkin-Elmer Model 303 Atomic Absorption spectrophotometer according to the procedure described by the manufacturer of the instrument (Anonymous, 1976). The method of Fiske and Subbarow (1925) was used to determine phosphorus. Total carbohydrate was determined by difference and caloric values were calculated according to Watt and Merrill (1963). An analysis of variance using the Duncan's Multiple-range test at the 5% level was performed on the data according to Steel and Torrie (1960). Duplicate determinations were run on each sample.

RESULTS & DISCUSSION

THE DATA PRESENTED in Table 1 show significant variation in the nutrient composition of corn tortillas, with the exception of ash, fat, and sodium. Tortillas from factory B had a lower pH value, but higher values for thiamin and riboflavin. Harris (1975) points out that the stability of thiamin and riboflavin is very sensitive to destruction by heat in alkaline media. Thus, these nutrient values could be related to the pH of the tortillas. The lower content of calcium for tortillas manufactured by factory B suggests a more thorough washing of nixtamal (cooked corn) after cooking in the lime solution. Although corn tortillas are lacking in some essential vitamines, they are a good source of other important dietetic nutrients such as fiber, calcium and potassium and have lower fat and sodium contents.

Significant differences exist in the nutrient content of flour tortillas (Table 2). Most of the differences noted could be explained by formulation variations. However, the

Table 1—Nutrient composition of corn tortillas[a]

Analysis	A	B	C	D	E	$S_{\bar{x}}$	Bread[b]
pH	9.0c	7.3a	8.7bc	8.2b	8.5bc	0.21	*
Moisture (%)	38.2a	38.6a	43.0b	40.5ab	42.3b	0.93	35.8
Ash (%)	1.1a	0.9a	1.0a	1.3a	1.0a	0.12	1.9
Fat (%)	0.9a	1.0a	1.0a	0.8a	0.9a	0.19	3.2
Fiber (%)	1.3a	1.3a	1.5b	1.4ab	1.7c	0.05	0.2
Protein (%)	5.8abc	6.0bc	5.5ab	6.1c	5.4a	0.15	8.7
Carbohydrates (%)[c]	52.7c	52.3bc	48.0a	49.9ab	48.7a	0.78	50.4
Caloric Value[d] (cal/100g)	235.6b	235.7b	217.1a	224.8ab	218.4a	3.75	269.0
Iron (mg/100g)	1.8c	1.4ab	1.2a	1.8c	1.5bc	0.10	2.4
Calcium (mg/100g)	210.0b	87.3a	180.4b	147.7ab	192.5b	20.0	70.0
Potassium (mg/100g)	124.0ab	154.7c	105.3a	139.9bc	104.5a	6.3	85.0
Sodium (mg/100g)	16.7a	17.2a	20.1a	21.5a	20.8a	3.2	507.0
Phosphorus (mg/100g)	52.8a	48.5a	56.5ab	63.2b	66.4b	3.7	87.0
Vitamin C (mg/100g)	*[e]	*	*	*	*	—	Trace
β-Carotene (mg/100g)	*	*	*	*	*	—	Trace
Thiamin (mg/100g)	Trace	0.22b	*	0.04a	Trace	0.02	0.25
Riboflavin (mg/100g)	0.06a	0.12b	0.11b	0.06a	0.06a	0.01	0.17
Niacin (mg/100g)	1.38d	1.32cd	0.85a	0.88ab	1.12bc	0.08	2.30

[a] Means with the same letter on same line are not significantly different at the 5% level by Duncan's multiple range test.
[b] Values for white enriched bread with 1.2% nonfat dry milk obtained from Watt and Merrill (1963).
[c] Calculated by the "carbohydrate by difference" method (Watt and Merrill, 1963).
[d] Caloric value calculated according to Watt and Merrill (1963) for corn meal.
[e] * = not detectable.

Table 2—Nutrient composition of flour tortillas[a]

	Tortilla Factory					
Analysis	A	B	C	D	$S_{\bar{x}}$	Bread[b]
Moisture (%)	25.2a	26.2ab	29.3c	27.0b	0.44	35.8
Ash (%)	2.1b	1.8ab	2.9c	1.7a	0.11	1.9
Fat (%)	10.0c	3.6a	8.1b	8.9b	0.31	3.2
Fiber (%)	1.6b	0.4a	1.5b	1.6b	0.08	0.2
Protein (%)	7.1a	8.1b	7.0a	7.1a	0.19	8.7
Carbohydrates (%)[c]	54.0b	59.0c	51.3a	53.9ab	0.76	50.4
Caloric Value[d] (cal/100g)	313.4c	282.0a	286.3a	303.4b	1.96	269.0
Iron (mg/100g)	1.9a	1.8ab	1.0a	1.5a	0.21	2.4
Calcium (mg/100g)	46.6b	27.2a	103.4d	65.4c	4.69	70.0
Potassium (mg/100g)	94.8ab	89.0a	110.5c	101.7bc	3.25	85.0
Sodium (mg/100g)	613.8b	531.9b	780.5c	371.2a	44.1	507.0
Phosphorus (mg/100g)	61.7a	98.3b	87.0ab	61.0a	8.45	87.0
Vitamin C (mg/100g)	*[e]	*	*	*	—	Trace
β-Carotene (mg/100g)	*	*	*	*	—	Trace
Thiamin (mg/100g)	0.41b	0.41b	0.18a	0.42b	0.02	0.25
Riboflavin (mg/100g)	0.24b	0.32c	0.07a	0.24b	0.02	0.17
Niacin (mg/100g)	3.70b	4.30cd	1.60a	4.01bc	0.16	2.30

[a] Means with the same letter on same line are not significantly different at the 5% level by Duncan's multiple range test.
[b] Values for white enriched bread with 1-2% nonfat dry milk obtained from Watt and Merrill (1963).
[c] Calculated by the "carbohydrate by difference" method (Watt and Merrill, 1963).
[d] Caloric value calculated according to Watt and Merrill (1963) for corn meal.
[e] * = not detectable.

lower values for thiamin, riboflavin and niacin in flour tortillas from factory C appear to be due to difference in the quality of flour used than to difference in formulation. It is very probable that factory C used nonenriched flour while factories A, B and D used enriched flour in the preparation of tortillas.

The results of this work show that corn tortillas are lower in protein, thiamin, riboflavin and niacin when compared to white enriched bread. Furthermore, a decrease in pH of the corn masa to 7.3 or below improves the retention of thiamin, riboflavin and niacin by the corn tortilla. Flour tortillas are comparable to white enriched bread when enriched wheat flour is used in the manufacture of this commodity. The wide variation shown in the nutrient composition of both corn and flour tortillas is due primarily to formulation differences in amounts of ingredients used in the formulations at the various tortilla factories. Tortilla manufacturers could improve the nutritive value of tortillas through quality control and through formulation by supplementation with nutrients known to be deficient and by reducing the levels of those known to be undesirable.

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A Research Note

Amylose Content and Puffed Volume of Gelatinized Rice

D. E. GOODMAN and R. M. RAO

ABSTRACT

A total of 113 experimental and commercial rough rice consisting of 6 short, 49 medium and 58 long grain types were used to determine the role of amylose and other selected properties on the puffing characteristics of gelatinized rice. Statistical analysis of the data on head rice yield, volume, length-width ratio, moisture of gelatinized dried rice, amylose and expansion volume showed that (1) head rice negatively correlated to amylose ($r = -0.48$), (2) length-width ratio positively correlated to grain type ($r = +0.95$) and expansion ($r = +0.62$) and, (3) expansion positively correlated with amylose ($r = 0.36$).

INTRODUCTION

MOST OF THE MEASURES of rice quality relate to either the amylose content of the rice kernel or the gelatinization temperature of the rice. Reports by Rao et al. (1952), Juliano et al. (1965), Webb et al. (1972) and Webb and Stermer (1972) indicated that amylose content of rice is considered to be the most important characteristic in the determination of cooking and eating quality of rice. The role of amylose in influencing the degree of puffing of parboiled rice was reported by Juliano (1975); wherein, he indicated a negative relationship between amylose content and the degree of puffing of parboiled rice. In other studies, amylose has been positively correlated to increases in cooked volume of rice (Juliano et al., 1965) and negatively correlated with increased puffed volume of cooked rice (Antonio and Juliano, 1973). Juliano (1975) noted that puffing was a complex concept, not merely the flashing off of the internal moisture of the kernels. He postulated several factors, e.g., amylose, moisture and compactness of the kernel content, probably work in concert affecting the puffing quality of rice.

Due to the lack of understanding about the interrelationships of the amylose and other physiochemical properties of rice and the puffability of rice, the industrial buyer has no other basis other than historical evaluation upon which to buy rice for puffing. Neither is the rice breeder any better equipped to breed new varieties of rice which would exhibit superior puffing characteristics. Therefore, this investigation was performed to investigate the extent to which amylose in concert with other physiochemical parameters of rice affect the degree of puffing of gelatinized rice.

MATERIALS & METHODS

A TOTAL of 113 rough rice samples consisting of 6 short, 49 medium and 58 long grain type varieties obtained over a 2-yr period (1979 and 1980) from rice experiment stations in Arkansas, Louisiana, Mississippi, and Texas were used in this study. The preparation of samples consisted of initially determining the moisture content of rough rice (Motomco Moisture Meter, Model 919). This was followed by hulling (using and adjusting McGill sheller for each grain type according to the U.S. Department of Agriculture Handbook (1976) milling (McGill number 2 mill), and grading (USDA approved rice grader with sizing device). The above resulted in white head rice samples to be used in subsequent investigations. The milled rice samples were analyzed for volume by the kerosene displacement method (Goodman, 1981), length to width ratio (Juliano, 1971). The white head rice samples were cooked (by adding 50g of rice in 400 mL of boiling water for 12-15 min for complete gelatinization). Samples were air dried (in drying racks) to 10-14% moisture. The moisture of the dried sample was monitored using the Delmhorst Model G-6. Upon reaching the desired moisture level, each sample was placed into a glass container and sealed, which allowed moisture levels to equilibrate. The equilibrated samples (10g of rice with known volume, using a graduating cylinder) were puffed in hot vegetable oil (246°C for 8 sec). The puffed rice was patted dry to remove excess oil and the bulk volume of the puffed rice was determined using a 250 mL graduated cylinder. The degree of puffing was determined using the formula: $X = F/I$ where X is the volumetric increase or degree of puffing, F is the final volume or the volume of the puffed rice, and I is the initial volume of the cooked and dried rice. The various statistical analysis procedures were performed on an IBM 370/3033 computer using the Statistical Analysis System software package from the SAS Institute, Inc. (Cary, NC).

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RESULTS & DISCUSSION

THE ANALYSIS OF VARIANCE of the experimental data and correlation analysis of degree of expansion of puffed gelatinized rice with head rice yield, rough rice moisture content, volume, length-width ratio, cooked and dried rice moisture, amylose and grain type are shown in Tables 1 and 2, respectively. Statistical analysis of percent head rice yields indicated that long grain type rice had significantly lower head rice yield than either short or medium types of rice. Several factors seem to be important in affecting the yield of head rice. Since milling involves the abrading of kernel against kernel, the longer, thinner kernels would tend to break more easily than the shorter, fatter kernels. Correlation analysis of expansion of rice (Table 2) with head rice yield showed no significant effect.

The volume of rice kernel is involved in the puffing of rice in that the amount of kernel that surrounds any moisture in the center of the grain affects at least two processing parameters: (1) the amount of thermal energy required to penetrate the kernel to flash any moisture at the center of the grain; and (2) the distance to which the moisture within the kernel must travel is driectly related to volume. Analysis of variance of the rice volume data (Table 1) indicated significant variation by type. Short grain varieties are shown to have volumes similar to those of both medium and long grain varieties. Correlation analysis of expansion of rice with volume showed no significant correlation. The lack of correlation with expansion suggests that there are other properties such as chemical forces binding the internal moisture that exert a greater influence on puffing than the physical orientation of the rice kernel.

The analysis of variance of length-width by grain type gave statistically distinct values for each grain type as shown in Table 1. Correlation analysis (Table 2) showed length-width ratio was significantly and positively correlated to expansion.

Table 1—Analysis of variance of head rice yield (HDYLD), rough rice moisture (HOHR), volume (V), length to width ratio (LWRATIO), cooked and dried moisture (HOHCK), amylose (AMY) and the degree of volumetric expansion (EXP) of gelatinized rice by grain type

		Mean						
Type	No. of samples	HDYLD (%)	HOHR (%)	V_3 (mm)	LWRATIO	HOHCK (%)	AMY (%)	EXP
Short	6	62.06[a]	10.83[a]	13.60[b]	1.96[c]	11.18[a]	11.13[c]	5.53[b]
Medium	49	58.10[b]	10.64[a]	13.83[a]	2.19[b]	11.27[a]	14.64[b]	5.83[b]
Long	58	50.89[c]	10.37[a]	13.13[b]	3.14[a]	11.73[a]	22.56[a]	6.73[a]

[a-c] Means within the same group with the same letter are not significantly different at P ≤ 0.05 using Duncan's Multiple Range test.

Table 2—Correlation analysis of degree of expansion of puffed gelatinized rice with head rice yield (HDYLD), rough rice moisture (HOHR), volume (V), length-width ratio (LWRATIO), cooked and dried rice moisture (HOHCK), amylose (AMY) and grain type

	Expansion (EXP)
Head rice yield (HDYLD)	r = −0.07
Rough rice moisture (HOHR)	r = −0.04
Volume (V)	r = −0.10
Length:width ratio (LWRATIO)	r = +0.62**
Cooked rice moisture (HOHCK)	r = +0.27*
Amylose (AMY)	r = +0.36**
Type	r = +0.65**

** Highly significant (P ≤ 0.01)
* Significant (0.01 ≤ 0.05)

The analysis of variance of amylose by grain type (Table 1) showed that amylose content varied significantly among short, medium, and long grain types. The reports (Juliano, 1970; Antonio and Juliano, 1973) that rice samples with high amylose content puffed poorly in relation to those samples with lower amylose content indicated amylose played a key role in thermal processing of cooked rice. In the present study, correlation analysis of amylose (Table 2) with expansion of cooked rice showed amylose to be positively related to expansion which differs from published results. However, multiple regression analysis showed amylose to be negatively related to expansion indicating that when more than two variables are acting together the partial contribution of each to the overall expansion may be greatly altered from those effects when the variables are acting independently.

In conclusion, the results of this investigation indicate that in addition to the amount of amylose, free moisture, surface area-volume ratio, length-width ratio and perhaps other physical and chemical characteristics of rice affect the degree of puffing of rice.

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A Research Note

Maillard Browning of Common Amino Acids and Sugars

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ABSTRACT

Common amino acids and amide derivatives have been ranked according to the intensity of Maillard browning formed when heated in an autoclave at 121°C for 10 min. under identical conditions, with each of the sugars D-ribose, D-glucose, D-fructose, α-lactose and sucrose. The amino compounds have been grouped into high, intermediate and low browning producing groups. Browning formed by heating a representative of each group with D-glucose, D-fructose and α-lactose at pH values ranging from 6–12 was studied. Maximum browning was obtained at a pH value of about 10 in all of the amino acid-sugar solutions tested.

INTRODUCTION

MAILLARD BROWNING REACTION involves the interaction of amino compounds, including all amino acids comprising natural proteins, and reducing sugars during thermal processing and storage of foods (Ellis, 1959). It is considered the most important browning reaction since it greatly influences the quality of processed foods. The reaction may be desirable as in baked, fried or roasted foods or undesirable as in concentrated and dried foods (deMan, 1980). In either case, it is important to study the factors affecting the intensity of Maillard browning.

Previous reports (Wolfrom et al., 1947, 1974; Katchalsky and Sharon, 1953; Schroeder et al., 1955; Lento et al., 1958; Willits et al., 1958; Underwood et al., 1959; Pomeranz et al., 1962; Kato et al., 1969) have shown that among the factors affecting the rate of Maillard reaction are the type of amino compounds and reducing sugars, and the pH of the medium. Some of these reports (Wolfrom et al., 1947, 1974; Schroeder et al., 1955; Lento et al., 1958; Willits et al., 1958; Underwood et al., 1959) dealt with the Maillard browning of some amino acids in the presence of only one or a few reducing sugars. Other studies (Katchalsky and Sharon, 1953; Pomeranz et al., 1962) reported Maillard browning of reducing sugars with only one or two amino acids. None of these studies has examined Maillard browning produced by each of the common amino acids constituting natural proteins and common reducing sugars under identical conditions. The different conditions used in the previous studies do not warrant ranking of common amino acids and reducing sugars according to the Maillard browning they produce upon thermal processing and/or storage. Because of these different reaction conditions, the results reported in previous studies are often contradictory. For example, Wolfrom et al. (1974), studying glucose-amino acid systems, reported that L-arginine yielded more intense Maillard browning than glycine, alanine or proline (L-lysine was not included). On the other hand, Willits et al. (1958), studying the effect of pH and type of amino acid on browning of sugar solutions, concluded that arginine, histidine, and other basic amino acids (except lysine), had no positive effect on browning. Willits et al. (1958) also stated that the presence of alanine or glutamic acid in the glucose solution did not cause a significant increase in the color produced relative to the control at any pH value.

The main objective of the present study was to compare the Maillard browning intensities of each of the amino acids present naturally in proteins and common reducing sugars under similar conditions. The effect of pH on the Maillard browning intensity was also studied.

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MATERIALS & METHODS

Reagents

All chemicals used in preparing reagents were of analytical grade. The amino compounds (L-isomers) and sugars were purchased from Sigma Chemical Company (St. Louis, MO). Stock solutions (0.05M) of each of the amino compounds and sugars were prepared for the study. Phosphate buffer solutions (0.05M) of pH 6.0 and 7.5, and 0.05M carbonate buffer solutions of pH 8.0, 9.0, 9.5, 10.0, 11.0 and 12.0 were also used.

Procedures

One mL of each of the 0.05M amino compound stock solutions was pipetted into medium size screw cap tubes. This was followed by 1 mL 0.05M sugar stock solution and 8 mL of the proper buffer solution. The top of each tube was covered with a small piece of aluminum foil and the tubes were then capped tightly. The tubes were shaken gently, autoclaved at 121°C for 10 min, then cooled with tap water. The absorbance of the cooled solutions was measured at 420 nm with a Beckman Model 24 spectrophotometer (Beckman Instruments, Irvine, CA).

RESULTS & DISCUSSION

THE CONDITIONS used for comparing the Maillard browning intensities of common amino acids and sugars were chosen from previous studies to yield measurable browning with all amino compounds for ranking. The final concentration of the amino compound was chosen to be as low as 0.005M to overcome the low solubility of some amino acids such as L-tryptophan and L-tyrosine and to obtain moderate browning intensities in the heated solutions without dilution.

The Maillard browning intensities resulting from heating the amino compounds and reducing sugars are presented in Table 1. These results indicated that the common amino acids and amides may be classified into three groups according to the Maillard browning produced when they are heated with common reducing sugars. The first group includes the high browning producing L-amino acids lysine, glycine, tryptophan and tyrosine. The second group includes the intermediate browning producing L-amino acids proline, leucine, isoleucine, alanine, hydroxyproline, phenylalanine, methionine, valine, and the amides L-glutamine and L-asparagine. The third group includes the low browning producing L-amino acids histidine, threonine, aspartic acid, arginine, glutamic acid and cysteine. This classification of the amino acids is valid with any of the common reducing sugars used in the present study. The results shown in Table 1 also indicate that relating Maillard browning to the basicity of the amino acid is not valid since the neutral amino acid L-glycine yielded similar browning intensity to that of the basic amino acid L-lysine and much higher browning intensity (about three times higher) than that of the basic amino acid L-arginine.

Table 1—Intensity of Maillard browning of common amino compounds and sugars[a]

Amino compound[b]	Absorbance at 420 nm[c] +D-Glucose	+D-Fructose	+D-Ribose	+α-Lactose
Lys	0.947	1.04	1.22	1.23
Gly	0.942	1.07	1.34	1.49
Trp	0.826	0.853	0.972	1.32
Tyr	0.809	0.857	0.951	1.06
Pro	0.770	0.783	0.792	0.876
Leu	0.764	0.747	0.895	1.11
Ile	0.746	0.797	0.870	0.986
Ala	0.739	0.792	0.945	1.06
Hypro	0.738	0.752	0.813	0.899
Phe	0.703	0.751	0.800	0.941
Met	0.668	0.669	0.828	0.888
Val	0.663	0.800	0.772	0.900
Gln	0.602	0.644	0.633	0.639
Ser	0.600	0.646	0.679	0.751
Asn	0.560	0.578	0.565	0.560
His	0.535	0.573	0.529	0.609
Thr	0.509	0.601	0.590	0.600
Asp	0.353	0.426	0.378	0.336
Arg	0.335	0.331	0.370	0.312
Glu	0.294	0.338	0.341	0.320
Cys	0.144	0.202	0.150	0.273

[a] Amino compound-sugar molar ratio of 1:1, final concentration of 0.005M in 0.04M carbonate buffer of pH 9.0. The solutions were autoclaved at 121°C for 10 min.
[b] All amino acids and amides were L-isomers.
[c] Average of two determinations.

The majority of the amino compounds yielded the highest browning with α-lactose followed by D-ribose, D-fructose and D-glucose (Table 1). Sucrose did not yield detectable browning with any of the amino compounds indicating that sucrose was not hydrolyzed under the conditions used. These results are in agreement with previous studies (Ellis, 1959; Pomeranz et al., 1962; Kato et al., 1969).

L-lysine, L-alanine and L-arginine were chosen as representatives of high, intermediate and low browning producing groups, respectively, for the effect of pH study. The sugars chosen were the aldose, D-glucose; the ketose, D-fructose; and the disaccharide, α-lactose. Preliminary results indicated that under conditions used in this study, no detectable browning was produced under pH 6.0; therefore, a pH range of 6-12 was chosen. The Maillard browning intensities resulting from heating the three amino acid representatives with D-glucose and D-fructose at various pH values are presented in Fig. 1A and 1B, respectively. Results obtained with α-lactose followed a similar trend to the other two sugars. The results (Fig. 1) indicated that Maillard browning intensity increased as the pH of the amino acid-sugar solution increased, with a maximum at a pH value of about 10.0, then decreased at higher pH values. This trend was common to all three amino acids heated with D-glucose or D-fructose. The increase in Maillard browning intensity as alkalinity increased has been previously reported (Schroeder et al., 1955; Underwood et al., 1959; Pomeranz et al., 1962; Kato et al., 1969; Wolfrom et al., 1974). However, none of these studies has shown a pH effect similar to that reported in the present study. This may be because most of these studies did not use pH values higher than 9.0. Even though common food products do not usually have such high pH values, the information obtained from this part of the study may be valuable in the processing of certain products such as caramels.

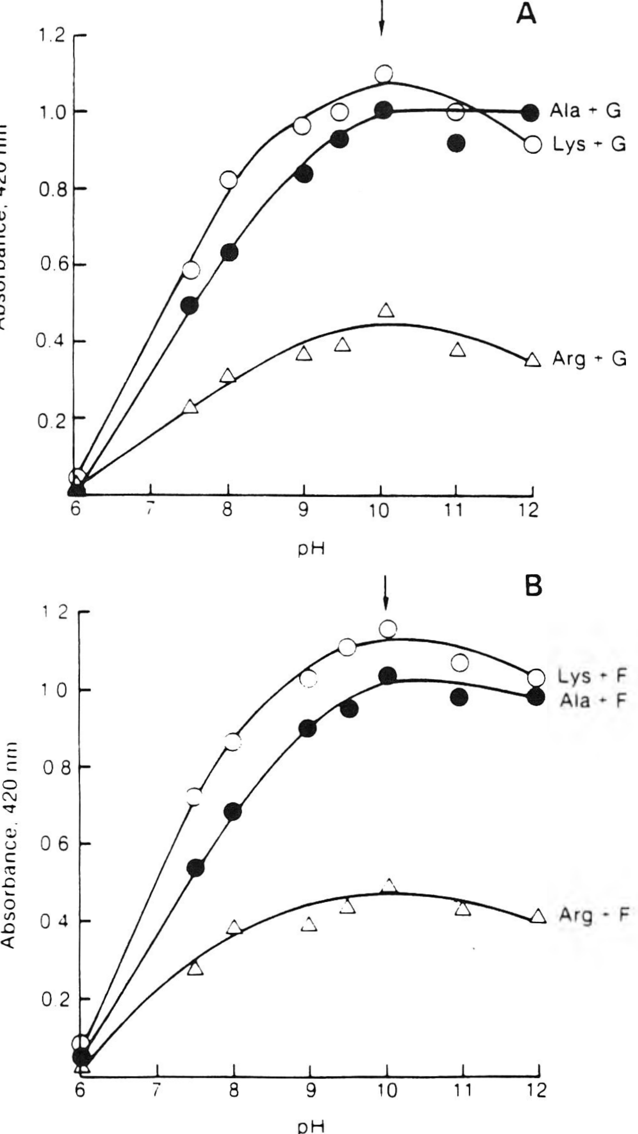

Fig. 1—Effect of pH on Maillard browning of L-lysine (Lys), L-alanine (Ala), and L-arginine (Arg) heated with D-glucose (G), A, and with D-fructose (F), B. The arrow indicates optimum pH.

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A Research Note

Volatile Components of Mushroom (*Agaricus subrufecens*)

CHU-CHIN CHEN and CHUNG-MAY WU

ABSTRACT

Volatile components of mushroom (*Agaricus subrufecens*) were extracted by the simultaneous distillation-extraction method. About 30 volatile components could be identified by GC and GC-MS analyses. Aromatic compounds, such as benzyl alcohol, benzaldehyde, benzonitrile, methyl benzoate, and a phenyl acetic acid-like compound, were the major volatiles (>85%) and are possibly the cause of "almond-like" aroma of this mushroom. Eight carbon compounds which are common to most mushroom volatiles make up only a minor part (ca 2%) of the total volatiles.

INTRODUCTION

VOLATILE COMPONENTS of edible mushrooms such as *Agaricus bisporus* (Cronin and Ward, 1971; Picardi and Issenberg, 1973), *Agaricus Campestris* (Tressl et al., 1982), *Lentinus edodes* Sing (Kameoka and Higuchi, 1976), *Boletus edulis, Cantharellus cibarius, Gyromitra esculenta, Lactarius trivialis, Lactarius torminosus, Lactarius rufus* (Pyysalo, 1976), and *Tricholomam atsutake* Sing (Yajima et al., 1981) are composed primarily of eight-carbon components such as 1-octen-3-ol, 2-octen-1-ol, 3-octanol, 3-octanone and 1-octanol., 1-Octen-3-ol, which is known as "mushroom alcohol," is the major volatile component of the above mentioned mushrooms. The formation of eight-carbon components is known from the results of enzymic reaction(s) using linoleic acid as the substrate (De Lumen et al., 1978; Tressl et al., 1982; Wurzenberger and Grosch, 1982; Chen and Wu, 1983).

Benzaldehyde, a volatile component, exists in many mushrooms and is a dominant aromatic component in *Agaricus campestris* (ca. 25%) (Tressl et al., 1982); however, there are no studies on its formation in mushrooms.

The present paper reports on the volatile benzyl components of the mushroom (*Agaricus subrufecens*).

MATERIALS & METHODS

FRESH MUSHROOMS were obtained from the cultivation house in the vicinity of Hsin-Chu, Taiwan. Mushrooms (300g) were blended with 700 mL distilled water for 5 min. Volatile components were extracted by simultaneous distillation-extraction (Römer and Renner, 1974) using glass-distilled pentane and diethyl ether (1:1) as extracting solvent. The volatile extract was dried over anhydrous Na_2SO_4 and concentrated tc minmum volume by using a Vigreaux column.

Gas chromatography (GC)

The GC was carried out on a Hewlett-Packard 5840A gas chromatograph, equipped with a flame ionization detector and a 50m × 0.25 mm (i.d.) WCOT stainless steel capillary column coated with PEG Carbowax-20M (Diasolid Co., Japan). The oven temperature was programmed linearly from 60°C to 160°C at 1.5°C/min, and then held at 160°C for 15 min. The injector and detector temperatures were 250°C. Carrier gas was helium at a flow rate of 1.2 mL/min. The peak area reported by the flame ionization detector was integrated through a built-in integrator (Hewlett-Packard 5840A GC terminal).

The linear retention indices of the volatile components were calculated using n-paraffin (C_8-C_{22}, Alltech Associates) as references (Májlát et al., 1974).

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Table 1—Composition of volatiles identified in the mushroom Agaricus subrufecens

Peak no.[a]	Component	I_k[b] CW-20M	%	Identification
1.	pentanal	930	+[c]	MS,GC.
2	hexanal	1078	0.1	MS,GC.
3	limonene	1168	+	MS
4	2-pentyl furan	1190	+	MS.
5.	3-octanone	1210	+	MS,GC.
6	amyl alcohol	1228	+	MS,GC.
7.	1-octen-3-one	1275	+	MS,GC.
8	octanal	1280	+	MS.
9	1-hexanol	1330	0.2	MS,GC.
10	2-methyl cyclohexanone	1393	0.1	MS.
11	1-octen-3-ol	1421	1.8	MS,GC.
12.	benzaldehyde	1473	9.3	MS,GC.
13	unk	1481	0.3	MS.
14.	benzonitrile	1567	0.7	MS.
15	methyl benzoate	1576	2.4	MS,GC
16.	2-octen-1-ol	1581	0.1	MS,GC.
17.	2-undescanone	1586	0.5	MS.
18.	menthol	1620	+	MS.
19.	benzyl formate	1635	0.2	MS,GC.
20.	unk[d] — 91(100),45(60),73(33), 134(27),92(22),65(17)	1652	0.6	MS.
21.	benzyl acetate	1664	0.3	MS,GC.
22.	unk[d] — 91(100),45(37),134(20), 73(19),92(18),90(10)	1670	0.3	MS.
23.	unk	1689	3.0	MS.
24	unk (phenyl acetic acid-like)[d] 90(100),136(90),91(88),65(25) 89(20)	1801	8.7	MS.
25.	benzyl alcohol	1841	66.3	MS,GC.
26	phenol	1943	0.3	MS.
27.	gamma-nonalactone	1980	0.1	MS.
28	2-pentadecanone	1988	+	MS.
29	neryl hexanoate	2017	0.2	MS.
30	5-methyl-2-phenyl-2-hexenal	2070	0.1	MS.

[a] Number refers to Fig. 1
[b] Calculated Kováts' indices
[c] Less than 0.1%
[d] Mass spectra data: M/z (relative intensity)

Gas chromatography-Mass spectrometry (GC-MS)

GC-MS was carried out on a Hewlett-Packard 5985B system, and operation parameters were as follows: carrier gas, helium; ionization voltage, 70 eV; electron multiplier voltage, 2200 V; ion source temperature, 200°C.

RESULTS & DISCUSSION

FIG. 1 SHOWS the gas chromatogram of the extracted volatiles of the mushroom, *Agaricus subrufecens*. A total of 30 components can be extracted by the simultaneous distillation-extraction method.

Table 1 shows the composition of volatiles identified in the mushroom. Identification was accomplished by comparing the mass spectra of the components with the published mass spectral data and their GC retention times with authentic samples (MSDC, 1974; Jennings and Shibamoto, 1980; De Brauw et al., 1981; Tressl et al., 1982).

Of the components identified, aromatic components were the largest group of all the isolated volatiles. Benzyl alcohol, at 66%, was the most abundant component; other major aromatic components included peak 24 (a phenyl acetic acid-like compound), benzaldehyde, benzonitrile and methyl benzoate. The existence of

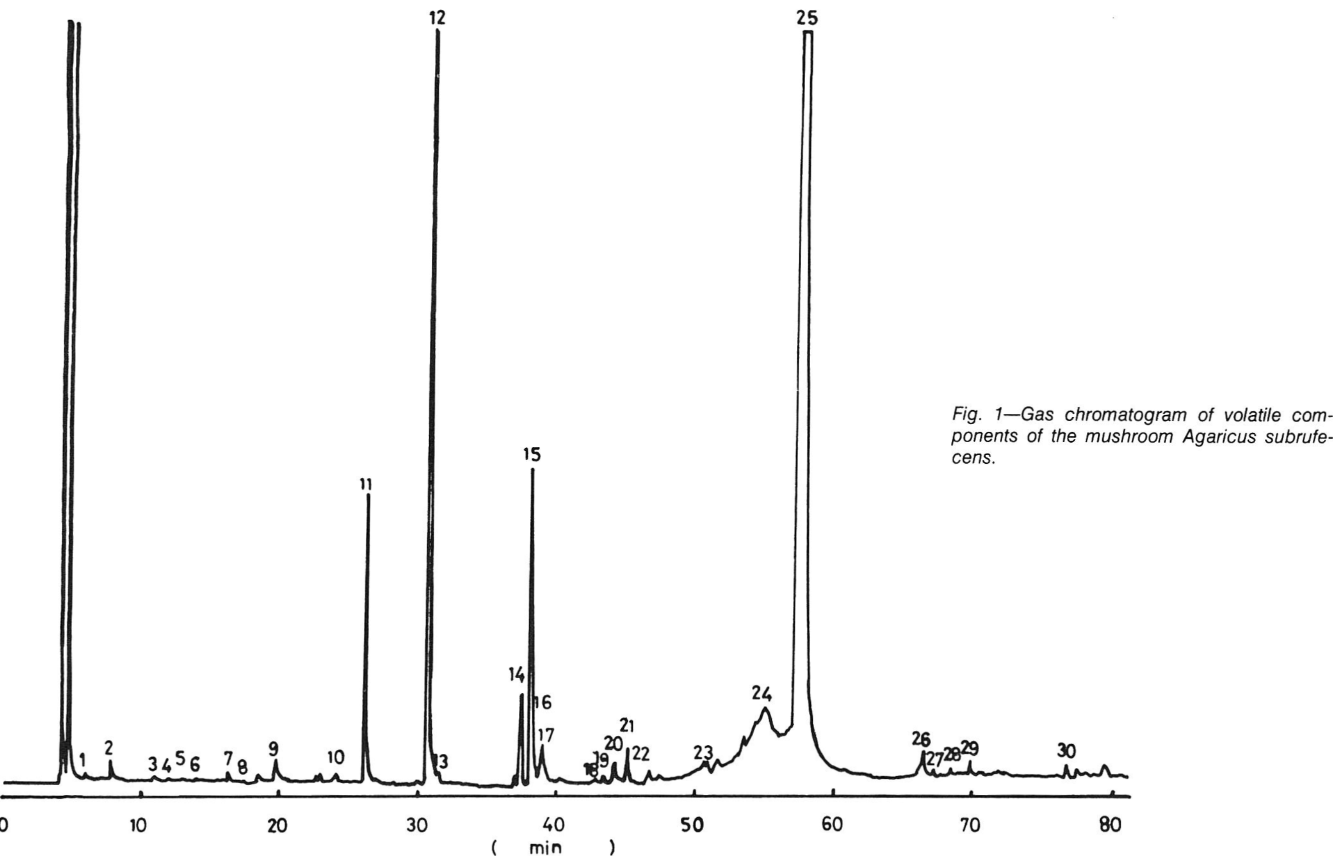

Fig. 1—Gas chromatogram of volatile components of the mushroom Agaricus subrufecens.

such a large amount of aromatic components may be the cause of the "almond-like" aroma during blending of this mushroom.

Eight-carbon components, long studied by many researchers and found in many mushroom volatiles, play a minor role in the volatiles of *Agaricus subrufecens*. The total concentration of eight-carbon components did not exceed 2%. Apparently, enzymic systems, which are responsible for the formation of eight-carbon components in other mushrooms, are either depleted or nonfunctional in *Agaricus subrufecens*.

The high content of aromatic compounds and their similarities in structure indicate that the aromatic compounds may have a common origin. It is possible that enzymic reactions are involved in the formation of these aromatic components. In *Agaricus bisporus*, the formation of benzaldehyde and benzyl alcohol could be increased to a significant extent if benzoic acid was blended together with fresh mushrooms (Chen and Wu, 1983), thus suggesting that there might be an enzymic system responsible for the reduction of benzoic acid or benzoic acid derivative (possibly benzoyl CoA) into benzaldehyde and benzyl alcohol. The existence of an alcohol reductase for the reduction of 1-octen-3-one into 1-octen-3-ol in *Agaricus campestris* (Tressl et al., 1981) and also in *Agaricus bisporus* (Chen and Wu, 1983) suggest the possible existence of a reductase system for aromatic components such as benzyl alcohol and benzladehyde in *Agaricus subrufecens*.

It is also interesting to note that all the aromatic components identified in this study seem to have a close inter-relationship, for example, methyl benzoate is the esterification product of benzoic acid; benzonitrile may be hydrolyzed to form benzoic acid; benzyl formate and benzyl acetate are the esterification products of benzyl alcohol; and peak 20, 22 and 24 are unknown aromatic components with the benzyl-group structure.

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A Research Note

Glucoamylase of *Amylomyces rouxii*

HWA L. WANG, E. W. SWAIN, and C. W. HESSELTINE

ABSTRACT

The production of glucoamylase by *Amylomyces rouxii*, a mold used in rice and cassava fermentations in the Orient, was demonstrated under solid substrate culture conditions. The enzyme purified by ammonium sulfate fractionation, gel filtration, and Sephadex ion exchange column chromatography appears homogeneous. *A. rouxii* glucoamylase is a glycoprotein and has an optimum pH around 4.5, optimum temperature 60°C, a molecular weight of 55,600 daltons, and K_m values of 15.8, 27.6, 16.8 mg/mL for soluble starch, glycogen, and amylopectin, respectively. Unlike the other fungal glucoamylases, which were found (from culture filtrates) to exist in multiple forms, *A. rouxii* glucoamylase, isolated from solid substrate fermentation, displayed only one form.

INTRODUCTION

GLUCOAMYLASE is capable of converting starch quantitatively to glucose; hence, it has considerable industrial importance. The enzyme is best known to be produced by species of *Aspergillus* and has been isolated from culture filtrates of *A. niger* (Lineback et al., 1969), *A. awamori* (Smiley et al., 1971), *A. oryzae* (Razzaque and Ueda, 1978), and *A. saitoi* (Takahashi et al., 1981). Also, glucoamylases of varying degrees of purity have been obtained from *Rhizopus delemar* (Takahashi et al., 1978), *Mucor rouxianus* (Tsuboi et al., 1974), and *Monascus kaoliang* (Iizuka and Mineki, 1977).

Amylomyces calmette is a monotypic fungus genus that has been found only in preparations of inoculum for rice and cassava fermentations in Asia, especially in China, Indonesia, and the East Indies (Ellis et al., 1976). The growth of this unique mold on starchy materials is near white with no cottony aerial growth. Consequently, it results in an attractive food product. In these fermentations, amylolytic enzymes are probably the predominant extracellular carbohydrases; however, the amylolytic enzymes of this genus have not yet been studied. This paper reports the production of glucoamylase by *A. rouxii* and also describes a purification procedure and some characteristics of the homogeneous enzyme.

MATERIALS & METHODS

Strains of *Amylomyces rouxii* were isolated at the Northern Regional Research Center from starters, ragi and chiu-yueh, (obtained from Indonesia and China) that are used for making fermented foods, tape, and lao-chao, respectively. Solid state fermentation was carried out in 250-mL Erlenmeyer flasks. In each Erlenmeyer flask, 20g commercial long grain rice and 20 mL water were mixed and allowed to stand at room temperature for 1 hr with frequent shaking to ensure even distribution of water. The cotton-plugged flasks were autoclaved at 120°C for 20 min, cooled to room temperature, inoculated, and then incubated at 28°C for 5 days without shaking. The fermented mass was homogenized, extracted with 100 mL water, and centrifuged. The supernatant was assayed for enzyme content. In preparing crude enzyme for purification, solid state fermentation was carried out in the same manner as stated above except that 2.8-L Fernbach flasks were used, and 160g rice, 40g wheat bran, and 200 mL water were added to each flask.

Glucoamylase activity was determined by measuring the amount of glucose released from gelatinized soluble starch solution. The enzymatic digestions were performed with 5 mL 4% starch in 0.05M acetate buffer, pH 4.5, and 1 mL enzyme solution at 55°C for 15 min. The reaction tube was immersed in a boiling water bath for 5 min. The resulting glucose was measured with a Technicon autoanalyzer either by the glucose oxidase method (Hill and Kessler, 1961) or by reduction of an alkaline ferricyanide reagent (Hoffman, 1937). One glucoamylase unit is defined as the amount of enzyme that liberates 1 mg of glucose in 15 min at 55°C. The specific activity of the enzyme was expressed as the number of units per mg of protein. Protein was determined by the method of Lowry et al. (1951).

Polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (SDS, 0.1%) was carried out at room temperature in pH 8.6 glycine-tris buffer for 16-18 hr at a constant current of 13.5 mA. Proteins were stained with coomassie blue. Carbohydrates were located by the periodate-Schiff base reaction following the procedure described by Fairbanks et al. (1971). Reverse phase-HPLC was carried out by the method of Bietz (1983).

RESULTS & DISCUSSIONS

FOUR STRAINS of *A. rouxii*, NRRL 2928, 3160, 3187, and 5866, were tested for their ability to produce glucoamylase. When these molds were grown on solid state substrate of rice at 28°C for 5 days, they produced 69, 123, 94, and 144 units glucoamylase per gram substrate. The yield is relatively low as compared with the known high glucoamylase producers, *Aspergillus awamori* NRRL 3112 and *Aspergillus niger* NRRL 3122. The latter produced about 756 and 2794 units enzyme per gram substrate, respectively, under the same growth conditions. However, the yield of enzyme was doubled when the rice substrate was enriched with 20% wheat bran.

Crude enzyme supernatant obtained from extracting rice-wheat mixture fermented with *A. rouxii* NRRL 5866 was first fractionated with ammonium sulfate. The fraction of 50-80% saturation was then subjected to gel-filtration on Sephadex G-100 column (2.5 x 36 cm) at 25°C. Two overlapping peaks absorbing at 280 nm were noted. The first peak, which had the larger molecular size, possessed the glucoamylase activity. After gel filtration, further purification was carried out on the DEAE-Sephadex A-50 column (2.5 x 50 cm). The enzyme was recovered from the peak eluted with the starting buffer of 0.01M phosphate at pH 6.0. The partially purified material was submitted next to chromatography on a CM-Sephadex C-50 column. There were two major peaks observed (Fig. 1), but only the peak that eluted with 0.5M sodium chloride in the buffer displayed glucoamylase activity. No other peak was eluted when sodium chloride was increased to 1M. These purification steps resulted in a 65-fold increase in specific activity of glucoamylase: 425 units/mg protein of the purified enzyme vs 6.5 units/mg protein of the concentrated crude extracts. SDS-polyacrylamide gel electrophoresis of the purified enzyme preparation showed a single band in gels stained by coomassie blue, and reversed-phase HPLC also

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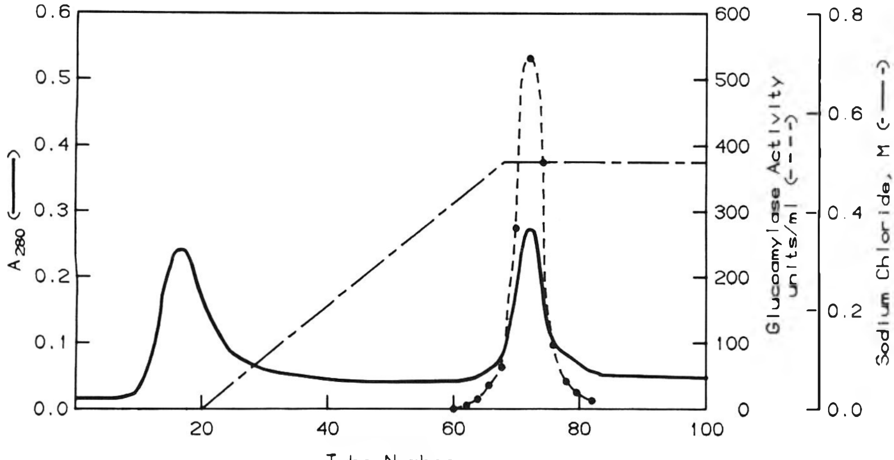

Fig. 1—Chromatography of Amylomyces rouxii *glucoamylase on CM-Sephadex C-50 column (2.5 x 50 cm); eluted with starting phosphate buffer, 0.01M, pH 6.0, 7 hr; followed by 16 hr-linear gradient, 0-0.5M NaCl in the same buffer; maintained at 0.5M NaCl for 10 hr; 5 mL/tube, 3 tubes/hr.*

gave one peak indicating the homogeneity of the enzyme preparation.

Amylomyces rouxii glucoamylase had a molecular weight of 55,600 daltons as estimated by polyacrylamide gel electrophoresis. The optimum pH of the enzyme at 55°C was found to be around 4.5. At this pH, the activity of the enzyme reached a maximum at 60°C. After that, there was a sharp decrease in activity.

Two observations suggested that carbohydrate was associated with the *A. rouxii* glucoamylase. SDS-polyacrylamide gels of the purified enzyme stained with either coomassie blue or with the periodate-Schiff reagent yielded an intense band at the same position on the gels. When the enzyme was incubated with Concanavalin A-Sepharose (from Pharmacia Fine Chemicals), no enzyme activity was found in the solution following removal of the gel.

The influence of substrate concentration on the rate of glucose formation with starch, glycogen, or amylopectin as substrate was studied, Data in Table 1 indicate that *A. rouxii* Glucoamylase interacted satisfactorily with all three substrates. The rate of glucose formation by the enzyme on soluble starch and amylopectin was similar, but it was slower on glycogen. The results were graphed on a Lineweaver-Burk plot and the resulting K_m values are 15.8 mg/mL for soluble starch, 27.6 mg/mL for glycogen, and 16.8 mg/mL for amylopectin. Smiley et al. (1971) reported that K_m values of glucoamylase isoenzymes isolated from several sources ranged from 13.7-25.5 mg/mL on glycogen and 10.9-19.2 mg/mL on soluble starch.

Thus, the characteristics of *A. rouxii* glucoamylase are similar to glucoamylase produced by many other molds. But unlike the other molds that have been reported to produce multiple forms of glucoamylase, *A. rouxii* was found to produce only one form.

Table 1—Effects of substrate concentrations on the rate of glucose formation by A. rouxii *glucoamylase with various polysaccharides*[a]

Substrate	Glucose formation mg/mL/15 min		
(mg/mL)	Starch	Glycogen	Amylopectin
1.6	0.85	0.56	0.90
3.2	1.61	1.07	1.66
4.8	2.25	1.56	2.44
8.0	3.31	2.25	3.59
12.8	3.95	3.08	4.25

[a] 35 units of enzyme/mL substrate.

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A Research Note

Effect of Ascorbic Acid on Color of Jellies

L. FREEDMAN and F. J. FRANCIS

ABSTRACT

Jellies, prepared from strawberry, blackberry, apple and orange juice, were fortified with ascorbic acid at 0, 35, and 70 mg/100 mL. After storage at room temperature, the jellies were sampled at intervals up to 32 wk for color measurement. The ascorbic acid did produce lighter products as shown by higher Hunter L values. The hues were shifted towards the yellow as shown by higher theta values. The ascorbic acid was well retained in the jellies. Whether the lightness and hue changes are desirable is probably a matter of individual preference.

INTRODUCTION

ASCORBIC ACID has been suggested for addition to jellies in order to prevent darkening and to improve nutritional value. There are logical reasons to expect ascorbic acid to improve the appearance of jellies. It might be expected to inhibit the oxidation of polyphenols, either naturally present or, produced by enzymatic browning prior to the heat treatment in the production of jelly (Haard, 1976; Richardson, 1976). The effect of ascorbic acid on nonenzymatic browning would probably darken the product since it could participate in the browning reaction (Tannenbaum, 1976).

The effect of ascorbic acid on the breakdown of anthocyanin pigments is well documented (Starr and Francis, 1968). This research was undertaken to investigate the color changes using systems which should demonstrate the above effects. The blackberry jelly was very dark due to the high concentration of anthocyanin pigments, and should show minimum color changes due to both darkening and pigment degradation. Strawberry jelly was chosen because it was medium in color and was very susceptible to browning. The apple jelly was pale pink due to the pigment from the skins and should show maximum color changes due to both pigment degradation and darkening. The orange jelly would show color changes due predominently to darkening since the carotenoid pigments present in orange juice are not sensitive to ascorbic acid.

MATERIALS & METHODS

Sources

The strawberry and blackberry jellies were made from juice pressed from frozen fruit obtained locally. The apple jelly was made from juice pressed from fresh apples. Commercial orange juice concentrate was used for the orange jelly.

The jellies were made in the conventional manner using a commercial pectin mixture (Sure-Jell) according to the manufacturer's instructions. For the blackberry and strawberry jellies, 800g juice, 1020g sucrose and 60g pectin were used. For apple jelly, the proportions were 1600g juice, 1820g sugar and 60g pectin. The orange jellies were made from 370g orange juice concentrate, 600g water, 900g sugar and 60g pectin. In each case, the pectin was added to the juice which was then brought to boiling temperature and the sugar added. After boiling 1 min, the jellies were poured into 224 mL (8oz) jars, and sealed with paraffin. The jars were stored at room temperature (22 ± 2°C) and sampled at 0, 2, 4, 8, 16, and 32 wk intervals.

Color measurement

The jellies were heated just enough to break the gel in order that the jelly could be poured into a 4 × 5 cm cell with a 2 cm light path without incorporating bubbles. The color was measured by transmittance using a Hunter D-25 colorimeter, standardized at L = 90, a = 0, b = 0 with distilled water.

The color data were interpreted in terms of L (lightness) and theta (a function of hue). The angle theta (θ) is actually the angle that a line joining a point in a Hunter a b plot with the origin, makes with the horizontal axis (Francis and Clydesdale, 1975). The function, θ, is most easily calculated from Hunter data as the angle whose cotangent is a/b. Thus a value of θ equal to zero would be a red color (θ representing 90, 180 and 270 would be yellow, green and blue, respectively).

Ascorbic acid

Ascorbic acid analyses were done at zero and 16 wk by the xylene indophenol method reported by Pepkowitz (1943) and modified by Starr and Francis (1968).

RESULTS & DISCUSSION

Color changes

Strawberry jelly. Fig. 1 shows definite changes in both lightness and hue for strawberry jelly. Both levels of ascorbic acid produced lighter jellies probably due to a combination of prevention of browning and degradation of anthocyanin pigment. Both

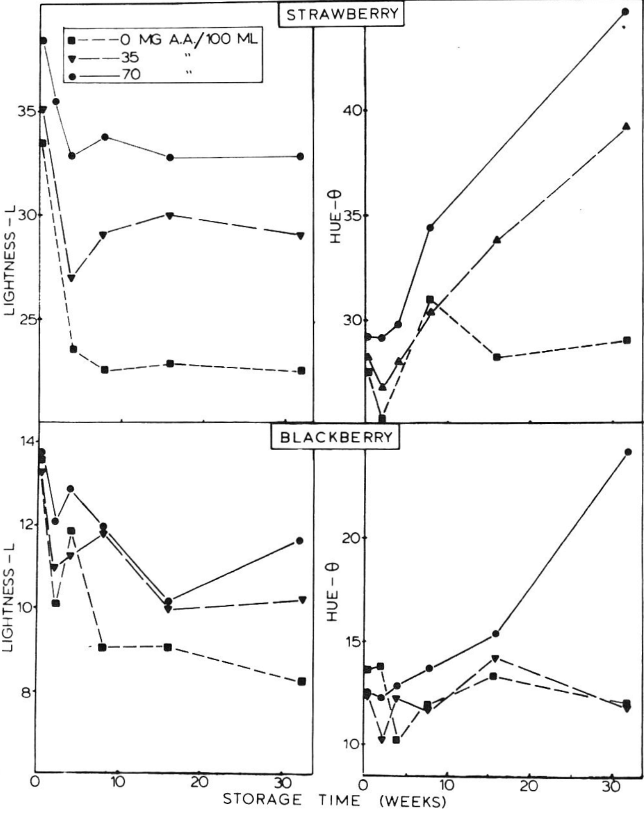

Fig. 1—Hue and lightness of strawberry and blackberry jellies during room temperature storage for 32 wk.

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levels of ascorbic acid produced changes in hue towards the yellow indicating a breakdown of anthocyanin pigment.

Blackberry jelly. Fig. 1 shows that both levels of ascorbic acid produced a lighter colored product after 16 wk of storage. The hue changes were similar for the zero and 35 mg levels of ascorbic acid and showed little change on storage. The 70 mg level produced a lighter colored product. The color changes with blackberry jelly were, as expected, much less than those with strawberry jelly.

Apple jelly. In Fig. 2, the lightness changes with increasing amounts of ascorbic acid were even greater than with strawberry jelly. This is probably due mainly to the degradation of the anthocyanin pigment. A small amount of pigment degradation in a dilute system has a much greater visual impact than the same amount of degradation in a system such as blackberry jelly with a high concentration of pigment. The large hue changes towards the yellow are probably due to the same reason.

Orange jelly. In Fig. 2, both the lightness and hue changed appreciably with all levels of ascorbic acid, but the effect of the ascorbic acid was not as pronounced as with the other jellies. Since there were no anthocyanin pigments in orange jelly, the changes were confined to the effects on browning. The jellies with the ascorbic acid were lighter and yellower than the controls.

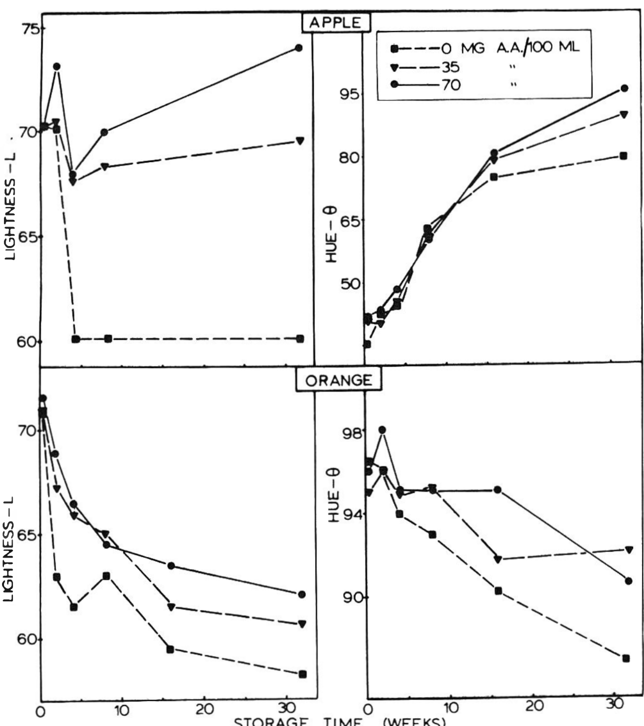

Fig. 2—Lightness and hue of apple and orange jellies during room temperature storage for 32 wk.

Ascorbic acid

The ascorbic acid was well retained in the jellies. With strawberry jelly, the three initial levels of ascorbic acid were 3, 34, and 76 mg/100 mL as compared with 3, 24, and 60 after 16 wk storage. With blackberry jelly, the initial and final levels were 0, 26, 68, and 0, 21, 60, respectively. With apple jelly, the values weere 0, 28, 78, and 0, 23, 70. With orange jelly, the values were 28, 56, 90, and 24, 50, 80. The high levels in the orange jelly reflected the high content of ascorbic acid in the orange concentrate. The claim for increased nutritional value due to the content of vitamin C are probably true. However, with the quantities of jelly normally eaten, this is not likely to be a significant source, unless the levels of fortification were quite high.

CONCLUSIONS

THE ADDITION of ascorbic acid to strawberry, blackberry, apple, and orange jelly did provide a lighter colored product, with hue changes towards the yellow. Whether these changes would be considered to have more, or less, marketing or consumer appeal would depend on individual preference.

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A Research Note

Distribution of Protein, Lycopene and the Elements Ca, Mg, P and N Among Various Fractions of Tomato Juice

P. LINDNER, I. SHOMER and R. VASILIVER

ABSTRACT

Samples of Israeli commercial tomato juice were analyzed for content of insoluble solids, and distribution of lycopene and elements Ca, Mg, P and N among various fractions. 75% of the insoluble solids were cell walls, still enclosing a part of intracellular material, and 25% were extracellular protein rich granules. More than 50% of the lycopene and insoluble N was found in the extracellular fraction of insoluble solids. About half the total amount of Ca was found in insoluble solids. Of the Ca in serum, about 70% precipitated with alcohol-insoluble solids (AIS) fraction. Almost all Mg in juice was soluble in serum and only 10% precipitated with AIS.

INTRODUCTION

THE INSOLUBLE SOLIDS of commercial tomato juice consist mainly of cell walls and heat-denatured cytoplasmic material. The cell walls are the major constituent which determine the gross viscosity (consistency) of the juice (Whittenberg and Nutting, 1957). The heat-coagulated cytoplasmic material which contains the tomato pigments, imparts the characteristic red color to the juice. A large proportion of this material appears in the juice as extracellular granules a few microns in size. Due to their size and density, the insoluble solids do not form a stable suspension in the serum. Their physical state can be considered as a precipitate which fills the whole volume of the sample (Shomer et al., 1984). The juice serum contains the soluble solids including soluble pectin, which is precipitated in the AIS fraction (Rouse and Atkins, 1955). The soluble pectin determines the serum viscosity, which also contributes to the gross viscosity of the juice (Hand et al., 1955). We report here on the distribution of lycopene and the elements Ca, Mg, P and N among various fractions of tomato juice, including cell walls, extracellular granules, serum and the AIS fraction precipitated from the serum.

MATERIALS & METHODS

COMMERCIAL, nonhomogenized tomato juice was obtained from a factory producing juice from tomatoes by a hot break process. Samples from 11 batches were selected for analysis.

Insoluble solids were separated from serum by centrifugation for 25 min at 27,000 × *g*. The insoluble solids were washed four times with distilled water to remove all soluble solids. To isolate the extracellular granule fraction, juice diluted tenfold with water was passed through a series of sieves, the finest of which was 200 mesh (0.074 mm). All the cells were retained by the sieves and the suspension of granules was centrifuged for 25 min at 27,000 × *g*. The granules were washed three times with water and redispersed in water to the desired volume. AIS was precipitated from serum according to the procedure described by Rouse and Atkins (1955). Analyses were carried out on samples of juice, serum, suspensions of isolated granules and solution of purified alcohol insoluble solids. Data for total insoluble solids were calculated as the difference between the results of juice and serum.

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Dry matter was determined by drying the insoluble solids at 70°C under reduced pressure of 170-200 mm Hg. Ca and Mg were analyzed by atomic absorption (Perkin-Elmer). N and P analyses were carried out on diluted Kjeldahl digests by a Technicon Auto Analyser system (1969). Lycopene was extracted from the insoluble solids with absolute ethanol and light petroleum ether (PE). The combined extracts were washed with water and the non aqueous phase was dried with anhyd. Na_2SO_4. Lycopene was determined in the PE at 474 nm using an absorption coefficient of $E_{1\%}^{1cm}$ = 3700 (Davis, 1976).

RESULTS & DISCUSSION

THE SAMPLES ANALYZED had Brix values of 5.6–6.4 and their pH was 4.0–4.3. Typical results for a single sample showing the distribution of lycopene and the elements N, Ca, Mg and P in whole juice and in its various fractions are shown in Fig. 1. The ranges of the results for the 11 batches tested are given in Table 1. Most of the nitrogen in the juice, 0.11–0.16%, is soluble in the serum. Only traces of precipitate were formed in serum after addition of trichloroacetic acid to a final concentraiton of 15%. The N soluble in the serum is mainly non protein N and is mainly amino acids N. The N in the insoluble solids can, however, be considered to consist mainly of protein N. The native tomato proteins are heat-coagulated by the prolonged heat treatment which is applied to the mass of broken tomatoes either immediately after breaking in the hot-break process, or after holding in the cold-break process (Gould, 1974). During the juice extraction process, part of the heat-denatured intracellular proteins together with other cytoplasmic components are expressed from the cells as small granules of a few microns in size. This extracellular granule fraction makes up only 24% of the total insoluble solids but contains 65% of the juice protein. On a dry weight basis, this fraction contained up to 50% protein (N × 6.25).

Less than 3% of the N soluble in the serum is precipitated with the AIS fraction. These small amounts of N could represent contamination of soluble N or small amounts of protein, <18 mg/100g juice, strongly interacting with pectin.

In fresh tomatoes, lycopene, which is the main tomato pigment (Hobson and Davies, 1981) is located in the chromoplasts and therefore in the juice it is found in the heat-denatured cytoplasmic material. About 66% of the lycopene in the juice was found in the extracellular granule fraction; the rest was found in the intracellular granules. In homogenized juice some of the lycopene is found in the serum obtained after centrifugation at 27,000 × *g* for 30 min. This is due to disintegration of part of the granules during homogenization, into submicron particles which are not precipitated under these conditions of centrifugation.

Practically all the Mg in the juice is soluble in the serum. Ca was found to be equally distributed between serum and the insoluble solids. A large proportion of the Ca soluble in serum, 60–80%, precipitated with the AIS fraction. The amount of Mg which precipitated with the AIS fraction was less than 15%. This reflects the greater affinity of pectic material for Ca, as compared with Mg (Doesburg, 1965). Most of the P in the juice is soluble in the serum.

The distribution of protein and lycopene among the various fractions of the juice reflect their distribution in the intact ripe

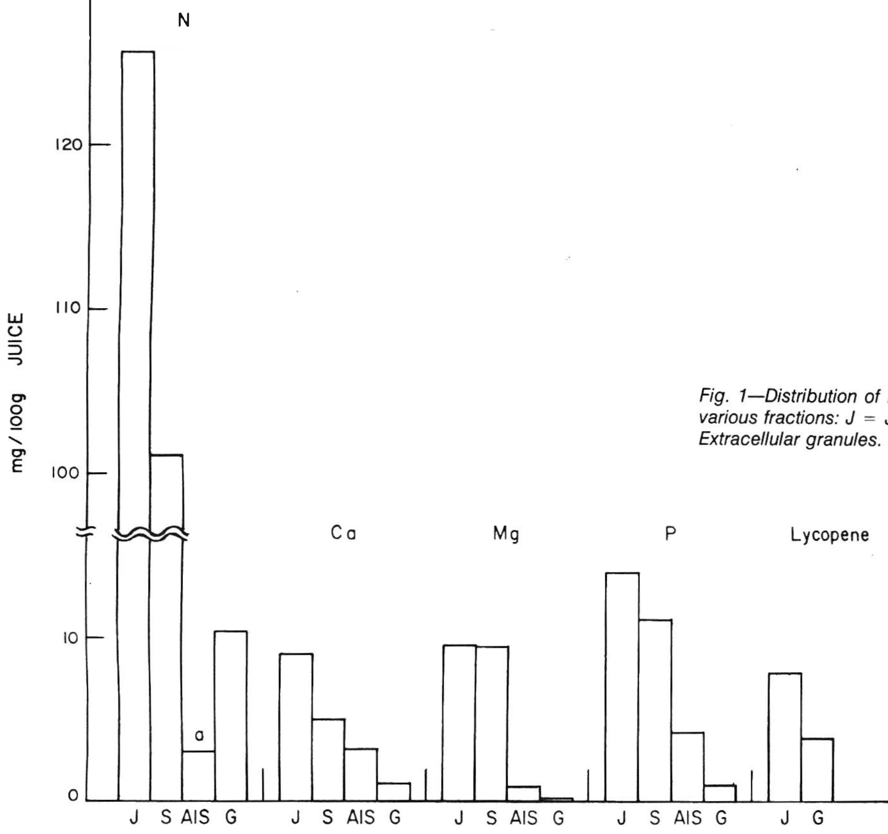

Fig. 1—Distribution of N, Ca, Mg, P and lycopene in whole juice and in its various fractions: J = Juice; S = Serum; AIS = AIS fraction of serum; G = Extracellular granules.

Table 1—Distribution of protein, lycopene, and Ca, Mg, P and N among tomato juice fractions[a]

		Serum		Insoluble solids		
	Juice	Total	AIS	Total	In extracellular granules	% In extracellular granules[c]
Insoluble solids	650–900	—	—	650–900	154–195	24
Ca	8.7–13.5	4.5–6.5	3.2–4.4	3.6–8.1	1.2–1.9	31
Mg	9.6–11.6	9.2–11.1	0.8–1.2	<0.6	<0.2	
P	12.5–17.5	10.4–15.1	<2	1.3–2.7	0.9–1.2	56
N	117–157	96–130	<3	15.5–24.5	10.4–14.0	66
Protein[b]	97–153	—	<18	97–153	65–87	66
Lycopene	5.8–9.0	—	—	5.8–9.0	4.6–5.8	67

[a] mg/100g juice
[b] Insoluble N × 6.25
[c] Mean

tomato. Since lycopene appears only in the chromoplasts which are located in the cytoplasm, we can assume that its distribution between the extracellular and intracellular insoluble solids in the juice represents the general distribution of coagulated cytoplasmic materials between these two fractions. Using this assumption, the total amount of insoluble solids of cytoplasmic origin can be calculated to be 250 mg/100g juice, or 34% of the total insoluble solids. Insoluble N (mostly protein N) and P are distributed similarly to lycopene (Table 1), their content in the cell wall is relatively low. A part of the P in the granule fraction of the insoluble solids has a constitutive role as a constituent of the phospholipids which are components of the various intracellular and plasma membranes. The low content of Mg in the granules can be explained by the degradation of chlorophyll pigments during maturation (Rabinowitch et al., 1975).

Using lycopene as a marker for the intracellular to extracellular distribution in the juice of material of cytoplasmic origin, the amount of Ca found in this material is estimated to be 1.9–2.5 mg/100g juice or 25–30% of the Ca in the insoluble solids. Most of the Ca in the insoluble solids is in the cell walls, bound to the pectic material. The level of Ca in the material of cytoplasmic origin presumably differs from its level in cytoplasm of intact tomato. Ca in unevenly distributed in the various internal parts of the cell (Rose and Lowenstein, 1975). The intracellular compartmentalization of Ca is destroyed on processing due to mechanical rupturing as well as heat coagulation. This allows redistribution of Ca and its binding to cytoplasmic constituents which tends to bind Ca, like proteins and phospholipids, as well as exchange of Ca between cytosol and cell wall. This effect is not expected for Mg which has a much lower affinity for pectic material in the cell walls, phospholipids and many cytoplasmic proteins (Carafoli and Compton, 1978).

The analysis presented in this publication emphasizes the importance of the distribution of the analyzed constituents among the various fractions of the juice. This information is valuable for assessing criteria for authenticity of juices, besides the usual criteria which are mainly based on the total contents and ratios of the various constituents. —*Continued on page 1218*

A Research Note

A Rapid Method for Determination of Limonin in Citrus Juices by High Performance Liquid Chromatography

P. E. SHAW and C. W. WILSON, III

ABSTRACT

The bitter component, limonin, was quantified in orange and grapefruit juices after preliminary separation on a C-18 cartridge, or by direct injection of the juice onto a C-18 or C-8 high performance liquid chromatographic (HPLC) column. The lower limit of detection for limonin was about 1 ppm on a 2.1 mm i.d. microbore column and 2 ppm on a 4.6 mm i.d. analytical column. In a few orange juice samples, the preliminary separation step was necessary to eliminate interfering substances prior to HPLC separation.

INTRODUCTION

LIMONIN is the major bitter component in processed navel orange juce and is one of two major bitter components in processed grapefruit juice. Various methods for quantitative determination of limonin in citrus juices were reviewed by Maier et al. (1977). More recently, an improved high performance liquid chromatographic (HPLC) method (Rouseff and Fisher, 1980) and an immunoassay method (Weiler and Mansell, 1980) have been developed for determination of this bitter compound in citrus products. The HPLC method requires a solvent extraction step that is difficult because of emulsion formation, and the immunoassay method afforded higher values than the HPLC method (Rouseff and Mansell, 1982). The thin-layer chromatographic (TLC) method of Tatum and Berry (1973) is rapid and simple, but quantitation depends on a subjective visual determination by comparison with limonin standards present on the same TLC plate. Thus, a rapid instrumental method for analysis of limonin in citrus products has yet to be developed.

The current study investigates the quantitative determination of limonin in orange and grapefruit juices by direct injection of the juice sample onto an HPLC column, or injection after preliminary separation on a C-18 "Sep-Pak" cartridge.

MATERIALS & METHODS

PROCESSED JUICE SAMPLES were either purchased at a local market or supplied by Florida Citrus World (Lake Wales, FL). Fruit for fresh juice samples were obtained from the Division of Plant Industry, Citrus Budwood Registration Bureau, Florida Department of Agriculture and Consumer Services, Winter Haven, FL. A standard solution of 100 ppm limonin was prepared by dissolving 5.0 mg of limonin crystals in 50 mL abs ethanol. Appropriate dilution with the HPLC elution solvent mixture afforded a limonin standard within ± 20% of the concentration in each juice sample analyzed (Shaw and Wilson, 1982). The limonin solution in abs ethanol was stable at −7°C for 3 months, but the diluted samples had to be prepared fresh daily. Use of five standards at levels of 2.5-22.5 ppm in 5 ppm increments at constant injection volumes showed a linear detector response (r = 0.992) for the range of samples analyzed (Rouseff and Fisher, 1980).

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Preliminary separation procedure

A C-18 "Sep-Pak" cartridge (Waters Associates, Milford, MA) was attached to a 10 mL syringe into which a small piece of silanized glass wool was packed to prevent plugging of the cartridge by unfiltered juice. The cartridge was washed with 2 mL acetonitrile and 5 mL water followed by 2.5 mL juice, 2.5 mL water, and 2.5 mL acetonitrile. The latter solvent removed the limonin that had been quantitatively absorbed from the juice sample. The acetonitrile wash was filtered through a 0.45 μm nylon 66 Millipore filter prior to analysis by HPLC. In one sample (Table 1) the solution was concentrated fivefold under N_2 prior to HPLC analysis. Limonin was quantitatively recovered from the cartridge when 2.5 mL juice with an initial limonin content of 2.9 ppm was spiked with either 5.1 ppm limonin (8.0 ppm total limonin) or 10.2 ppm limonin (13.1 ppm total limonin) and separated on the C-18 cartridge followed by HPLC analysis of the acetonitrile extract. Recovered limonin was 8.2 ± 0.4 ppm and 13.0 ± 1.0 ppm, respectively, (Table 1). The spiked samples were prepared by addition of 170 mL freshly prepared solutions of 6.0 ppm or 12.0 ppm of limonin in water (from 6.0 mg or 12.0 mg of limonin in 2.0 mL of acetonitrile diluted to 1000 mL with water) to 30 mL of 54°Brix frozen concentrated grapefruit juice.

Analytical methods

Quantitative TLC determinations were carried out by the procedure of Tatum and Berry (1973) with 10% sulfuric acid in ethanol as the spray reagent, except that the developing solvent was 100-50-50-3 benzene-chloroform-ether-acetic acid, which was developed by Tatum (1982). Quantities for TLC values in Table 1 are averages of determinations made by three staff members by the procedure of Tatum and Berry (1973). Identification of HPLC peaks corresponding to limonin, nomilin and sinensetin were made by collecting peaks from fresh navel orange juice from the C-8 column and spotting them on a TLC plate as above with standards spotted on adjacent bands. Similarly, limonin and 5[(6,7-dihydroxy-3,7-dimethyloctenyl)oxy]-psoralen were identified by isolation from reconstituted frozen concentrated grapefruit juice from a microbore C-18 column.

HPLC analyses were performed with a Perkin-Elmer Model Series 2 pump, a Rheodyne Model 7125 injector, a Model LC-85 variable wavelength spectrophotometric detector set at 207 nm, and a Hewlett-Packard 3390A recording integrator. The eluting ternary solvent ratio of acetonitrile-tetrahydrofuran-water (v/v/v) was 17.5-17.5-65 for the C-18 columns and 17.5-15-67.5 for the C-8 column. The C-18 columns used were a Brownlee 5 μm microbore (2.1 mm x 22 cm) column or analytical (4.6 mm x 10 cm) column with a 2.1 mm x 4 cm Brownlee 5 μm C-18 guard column attached and a 5 μL loop in either case. The flow rate was 0.2 mL/min for the microbore column and 0.5 mL/min for the analytical column. The C-8 column was a Perkin-Elmer 5μ analytical (4.6 mm x 12.5 cm) column with a 4.6 mm x 4 cm Brownlee 5μ C-8 guard column and a 20 μL loop at a flow rate of 1.5 mL/min. In all cases where juice was directly injected onto the column, a Waters 2 μm precolumn filter was used ahead of the guard column.

Quantitative determinations in Table 1 were made by comparison of average values of peak areas for triplicate determinations compared to averge values for triplicate determinations of limonin standards run the same day. Each standard was prepared so that peak area was within ± 20% of that for the juice sample or extract (Shaw and Wilson, 1982). Coefficients of variation for samples containing about 5, 10 and 15 ppm of limonin determined on 10 consecutive runs for each sample are shown in Table 1. For triplicate runs of juice sample used to obtain the HPLC values in Table 1, the average coefficient of variation was about 9%. This variation was generally within the tolerable limit for each concentration determined by Horwitz (1982).

Table 1—Limonin content in orange and grapefruit juices

Juice sample	ppm Limonin determined by:				
		HPLC[a]			
	TLC	MB	SP+MB	Anal	SP+C8
Orange					
Reconstituted navel conc	18	14.6	15.5(6)[b]	19.4[c]	—
Fresh navel	10	10.8	—	8.8	11.4
Canned	4	N.D.[d]	5.0(9)[b]	—	—
Canned	3	2.5	—	—	—
Reconstituted conc	3	N.D.	1.8	—	—
Reconstituted conc	2	N.D.	2.0;2.0[e]	—	—
Grapefruit					
Reconstituted conc	16	—	16.9	—	16.1
Reconstituted conc	10	9.4	9.7(6)[b]	9.6[c]	—
Reconstituted conc	8	—	—	8.3	—
Reconstituted conc	7	—	—	7.3	—
Glass-packed	5	3.8	—	4.0	5.4
Canned	4	4.6	—	5.3	—
Reconstituted conc	4	3.9	—	—	—
Reconstituted conc	3.5	—	2.9	—	—
Spiked with 5.1 ppm limonin			8.2		
Spiked with 10.2 ppm limonin			13.0		
Carton	3	2.8	—	—	—
Reconstituted conc	2	2.0	—	—	1.7
Fresh Royal	1.5	1.4	—	—	—

[a] MB = Microbore C-18 column; SP+MB = Sep-Pak separation and microbore C-18 column; Anal = 4.6 mm i.d. analytical C-18 column; SP+C8 = Sep-Pak separation and 4.6 mm i.d. analytical C-8 column.
[b] Coefficient of variation (%) determined on values from 10 consecutive runs.
[c] Value was 0.1 ppm higher than this when Sep-Pak purification step was included.
[d] N.D. = Not determined because of interfering peak.
[e] Sample concentrated fivefold before HPLC analysis.

RESULTS & DISCUSSION

LIMONIN in navel orange and grapefruit juices was quantified using a microbore (2.1 mm i.d.) C-18 column and analytical C-8 and C-18 columns when ternary solvent mixtures containing tetrahydrofuran, acetonitrile and water were used (Table 1). In most samples, limonin could be quantified by direct injection of the whole juice, but a few orange juice samples required preliminary separation on a C-18 "Sep-Pak" cartridge to remove an unknown substance that interfered with limonin analysis when direct injection of juice was used. The rapid and simple preliminary separation could be used on all samples to protect the column and guard column from possible contamination by solid juice particles. Quantitative recovery of limonin from the Sep-Pak cartridge was shown by analysis of a grapefruit juice sample spiked with a known quantity of limonin. Results from HPLC analyses were compared to those obtained by TLC on the same samples (Tatum and Berry, 1973).

The ternary mixtures separated limonin from sinensetin in orange juice and from 5[(6,7-dihydroxy-3,7-dimethyloctenyl)oxy]-psoralen in grapefruit juice (Fig. 1). Two-solvent mixtures of acetonitrile-water, tetrahydrofuran-water or methanol-water failed to achieve these separations which are necessary for quantitative determination of limonin. The ternary mixtures also separated another bitter component, nomilin, from limonin. Nomilin eluted at about 4.5 min after limonin on the C-8 column (Fig. 1a) and about 3.5 min later on the microbore C-18 column (Fig. 1b). Juice samples could not be filtered through a 0.45 μm filter to protect the column prior to HPLC analysis or prior to separation on a Sep-Pak cartridge because the filtration step removed about 50% of the limonin.

This reversed phase HPLC technique permited rapid quantification of limonin in orange and grapefruit juices at or above the 1 ppm level on a 2.1 mm i.d. microbore column or the 2 ppm level on a 4.6 mm i.d. analytical C-8 or C-18 column. The analytical column required an injection volume four times larger than that required with the microbore column to achieve this level of sensitivity (Bowermaster and McNair, 1983). The procedure took about 15 min for each run and an additional 5 min for Sep-Pak purification. Use of this purification step removed particulate matter, permitted analysis of some samples that showed interfering peaks by direct injection, and allowed concentration of some samples for increased sensitivity.

Advantages of the microbore C-18 column were greatly reduced solvent usage per analysis and smaller sample size required. The analytical C-8 column offered better resolution from the solvent front, but the negative peak at 8 min preceding the limonin peak (Fig. 1a) required delay of electronic integration until after the negative band eluted to obtain accurate quantitative results. Quantitation of the bitter component, nomilin, would be difficult by this procedure because of the relatively long retention time, small peak size, and incomplete separation from interfering components in grapefruit juice (Fig. 1b).

This rapid and simple HPLC method has potential for routine analyses to determine if juice samples meet the quality standard that requires an upper limit of 5 ppm of limonin in grapefruit juice processed in Florida from August 1 to December 1 (Florida Dept. of Citrus, 1975).

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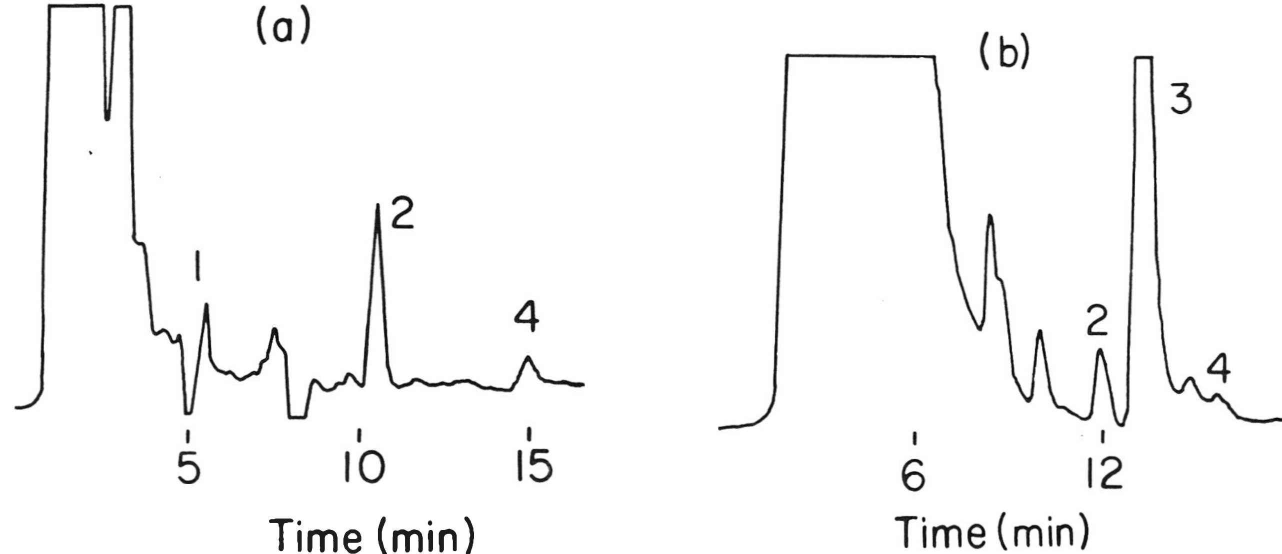

Fig. 1—(a) Fresh navel orange juice after Sep-Pak purification and separation on a 4.6 mm x 12.5 cm C-8 column at 1.5 mL/min. (b) Concentrated grapefruit juice reconstituted to single-strength and separated on a 2.1 x 22 mm C-18 column at 0.2 mL/min. Peak identification: 1, sinensetin; 2, limonin; 3, 5[6,7-dihydroxy-3,7-dimethoxyoctenyl)oxy] -psoralen; 4, nomilin.

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PROTEIN/LYCOPENE/MINERALS IN TOMATO JUICE . . . From page 1215

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A Research Note

Thermal Diffusivity of Papaya Fruit (*Carica papaya* L., Var. Solo)

CHARLES F. HAYES

ABSTRACT

The thermal diffusivity of papaya pulp is measured to be 1.52×10^{-3} cm^2/s and found to be in agreement with the value predicted by the Riedel equation within the uncertainty of the measurement. The thermal diffusivity of papaya seeds is measured to be 1.60×10^{-3} cm^2/s.

INTRODUCTION

BEFORE PAPAYA may be shipped to the Mainland from Hawaii the fruit must first be treated to prevent the transport of viable fruit fly infestation. The current treatment involves fumigation with ethylene dibromide (EDB). However, the Environmental Protection Agency has ruled that after September 1, 1984, EDB may no longer be used. One of the proposed alternative treatments (Hayes et al. 1984) involves heating the papaya to a level which would kill organisms in the fruit without damaging the fruit itself. It is important, therefore, to know the thermal diffusivity of papaya for an adequate evaluation of such an EDB alternative.

MATERIALS & METHODS

A SOLID CYLINDER of aluminum was constructed with a diameter of 7.81 cm and a length of 14.6 cm. The ends were cemented with a 2.3 cm layer of plastic foam to insure radial heat flow. A 5.2 mm diameter hole was drilled through one end of the cylinder along the symmetry axis to the center, to accomodate a YSI precision thermistor. The thermistor was connected to the input of a Keithley 177 DVM in the resistive mode. The analog output of the DVM was connected to the 14 bit analog input of a microcomputer which recorded temperature as a function of time. The cylinder, originally at 23°C, was placed in the center of a 0.8m x 0.5m x 1.4m tank of water maintained at 49°C. The upper foam layer extended just above the water surface. The water in the tank was continuously circulated by a pump with water emerging from the output hose, diameter 3.4 cm, at the rate of 1.8 liters per second. The location of the cylinder was approximately 20 cm in front of the hose opening. Qualitatively, the water surrounding the cylinder therefore could be described as highly agitated.

For an infinite cylinder the time response of the central temperature change is given by (Gaffney et al., 1980; Carslaw and Jaeger, 1959)

$$T_x = \frac{t_x - t_\infty}{t_i - t_\infty} = \sum_{n=1}^{\infty} \frac{2\exp(-\lambda_n^2 \alpha\theta/a^2)}{\lambda_n J_1(\lambda_n)} \quad (1)$$

where t_x is the temperature at time θ after the surface is given a temperature t_∞ and t_i is the initial temperature. $J_1(\ )$ is the first order Bessel function of the first kind, a is the radius, α is the thermal diffusivity and λ_n is the nth order solution of

$$\frac{ha}{K} = \frac{\lambda_n J_1(\lambda_n)}{J_o(\lambda_n)} \quad (2)$$

where h is the surface heat transfer coefficient, K is the thermal conductivity and $J_o(\ )$ is the zero order Bessel function of the first kind.

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After sufficient time, the higher order terms in Eq. (1) will be negligible resulting in

$$\ln T_x = -(\lambda_n/a)^2 \alpha\theta + \ln[2/\lambda_1 J_1(\lambda_1)] \quad (3)$$

which is linear in θ.

Using a linear regression, the values of T_x and θ for the aluminum were fitted to Eq. (3) from 10 sec after submersion to 20 sec after submersion. Values were taken every 0.25s. There were 10 repetitions.

Papayas were obtained from the Puna section of the island of Hawaii. Locally this type is called "Kapoho." Its pulp is more yellow and less pink than "Sunrise" papayas which are also shipped to the Mainland. The pulpfrom several papayas or the seeds including the placental membrane was placed in a cylindrical copper shell of outside diameter 7.74 cm, length 16.6 cm and wall thickness 1.6 mm. One end of the shell was sealed with copper. The other end was fitted with an aluminum cap, a rubber o-ring making a water tight seal. The ends of the container were thermally insulated from the pulp by plastic foam of 2.3 cm thickness. The thermistor was accommodated by a hole in the center of the top of the cap with a water tight seal provided by a second o-ring. The copper cylinder containing either pulp or seeds was initially brought to thermal equilibrium by submersion for several hours in 42.8 ± 0.8°C water, where the ± refers to the standard deviation among the 36 runs. It was then placed in the tank as the aluminum had been with the water temperature maintained 49.2 ± 0.7°C. An average of seven repetitions were taken on each of three pulp samples and an average of five repetitions on each of three seed samples. The experimental values of T_x were taken each minute for 50 min starting 10 min after submersion. A best fit for α was made to Eq. (1) for n = 1 to 5 with the λ_n defined by

$$J_o(\lambda_n) = 0 \quad (4)$$

Cutting off the series at n = 5 is more than adequate since by the time T_x has dropped to 0.9, around 10 min after submersion, the n = 5 term becomes smaller than the leading terms by a factor of approximately 10^9. The water content of the papaya pulp was determined by the difference in weight before and after drying.

RESULTS & DISCUSSION

THE LINEAR REGRESSION of the fit to Eq. (3) for the aluminum data had an average coefficient of determination of 0.99990 which justifies omitting all but the n = 1 term 10s after submersion. A best fit of the coefficient of θ in Eq. (3) gives

$$\lambda^2\alpha/a^2 = 0.1395 \pm 0.0005\ /s \quad (5)$$

where the ± refers to the standard deviation among the 10 repetitions. Eq. (2) now yields for the aluminum

$$h = 9{,}528\ W/m^2\ {}^\circ K \quad (6)$$

Assuming this same value of h applies to the papaya samples, the percent error incurred in the determination of α from the replacement of Eq. (2) by Eq. (4) is around 0.3% (Gaffney et al., 1980, Fig. 3).

The value of α as determined by the best fit of the data was $1.52 \times 10^{-3} \pm 0.11 \times 10^{-3}$ cm^2/s for pulp and $1.60 \times 10^{-3} \pm 0.165 \times 10^{-3}$ cm^2/s for the seeds. The water content was found to be 87.6% ± 1.3% for the pulp.

The Riedel Equation (Gaffney et al., 1980) is an empirical equation relating thermal diffusivity to water content of various food products: *—Continued on page 1221*

A Research Note

Effects of Titratable Acidity and Carbonation Level on the Preference of Clarified Pineapple Juice

J. D. BARANOWSKI and S. S. PARK

ABSTRACT

The titratable acidity (TA) of clarified pineapple juice was adjusted to 0.65, 0.75, 0.85, and 0.95% through ion-exchange treatment followed by the addition of citric acid. Each lot of juice was carbonated to three levels of carbon dioxide content (2.5, 3.0 and 3.5 gas:liquid volumes). Preference panels were conducted on these juices, holding either TA or gas volume constant and varying the other parameter. Although few statistically significant differences were found, an overall preference for a 0.75% TA, low carbonation product was observed.

INTRODUCTION

THE PERCEIVED FLAVORS of beverages are known to depend upon several chemical attributes of the products, including titratable acidity (TA), pH, soluble solids, and the soluble solids/TA (Brix/acid) ratio (Sweeney et al., 1970). Valdes et al., (1956a, b) in their extensive study on flavor, reported that sucrose enhanced the apparent flavor intensity of both aqueous solutions and fruit nectars, and that there was an optimum level of flavor enhancement by sucrose which, if exceeded, would interfere with flavor perception. Addition of organic acids enhanced the flavor of sweetened nectars, but the Brix/acid ratio was also found to be an important determinant of apparent flavor intensity. Nagel et al. (1982) found that even small differences in pH or TA had a marked influence on the preference of dry white wines. Although little acid or pH adjustment is made in juices, pH and TA are commonly adjusted in wines.

It is also known that carbonation of beverages enhances flavors. This effect is partly due to the increased acidity of the product, but in part is due to the unique zest, sparkle and taste of the carbon dioxide itself. Although other sparkling, i.e. carbonated, fruit juices have been on the market for over a decade, pineapple juice has not been available in this form. The purpose of this investigation was to develop a sparkling pineapple juice and to then determine the effects of different levels of titratable acidity and carbonation on its preference.

METHODS & MATERIALS

Preparation of juice

Commercial canned pineapple juice was filtered by suction through Whatman #1 filter paper after addition of 1% Celite filter aid (W/V) (Johns Manville, NY). The filtered juices were blended and 100 ppm SO_2 (as potassium metabisulfite) were added. Duolite A-7 [Diamond Shamrock, Redwood City, CA, (free base form)] was washed extensively and packed into a 3 × 25 cm column. Roughly 1 liter of clarified juice (TA of 0.69% expressed as citric acid) was applied to this column, at a flow rate of 20 mL/min. Sufficient 6N sodium hydroxide was added to this juice to restore its pH to the original 3.4, resulting in a TA of 0.23%. Treated juice was blended with untreated juice to yield 32 liters of clarified 13.5 °Brix juice with a TA of 0.65%. This juice was divided into four equal lots, which were adjusted to TA levels of 0.75%, 0.85% and 0.95%, by adding 30% (W/V) citric acid solution (pH 3.4). Actual TAs of the adjusted lots were verified after citric acid addition to insure that the desired TA had been obtained.

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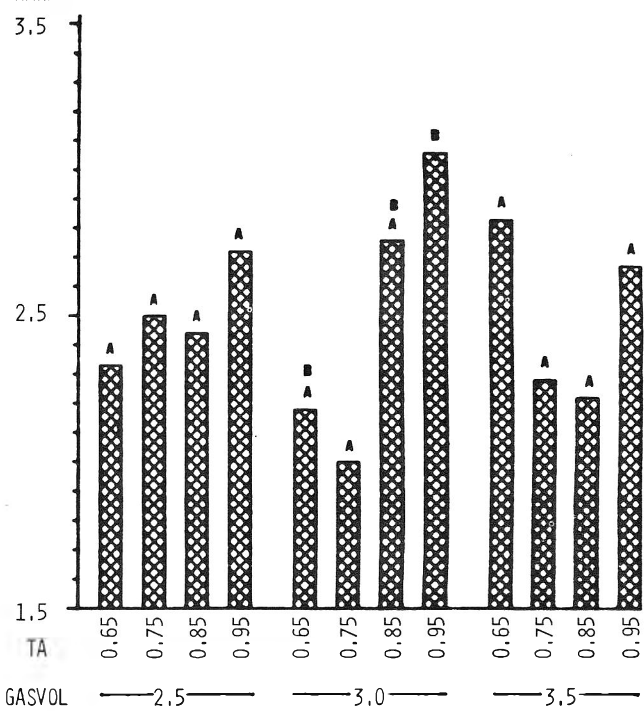

Fig. 1—Mean rank by taste panel of carbonation level effect on preference of clarified carbonated pineapple juice at constant TA. Within a TA, mean ranks are significantly different (P = 0.05) if they do not share a common letter.

Carbonation was accomplished with a Zahm and Nagel (Buffalo, NY) Pilot Plant carbonator at 4°C. For each TA level, 8 liters of juice were placed in the carbonator, and sufficient carbon dioxide (U.S.P. medical pure, Big Three Industries, Honolulu, HI) was charged into the tank to provide 2.5 volumes gas/volume liquid. The gas was bubbled through the tank for 15 min prior to filling eight 280 mL screw cap soda bottles. The pressure in the tank was increased to impart 3.0 volumes gas/volume liquid. After 15 min of equilibration, 8 more bottles were filled. Gas pressure was again increased to give 3.5 volumes gas/volume liquid, the tank was equilibrated for 15 min and a final 8 bottles of juice filled. Tank pressure was then released and the equipment readied for carbonation of the other TA juices.

Taste panel and statistical analysis

Seven panels were conducted on the prepared products. Eighteen untrained persons from the Depts. of Food Science & Human Nutrition, and Horticulture, Univ. of Hawaii, served as taste panelists. Panels were conducted in partitioned booths with fluorescent lighting and water was supplied for rinsing the mouth. At each session, one of the three carbonation levels, or the four TA levels was held constant and the other parameter varied. Each person participated in the entire study, except that one taster was absent for one panel. All samples were kept at refrigeration temperatures, and were tasted within 15 min of pouring. Roughly 30 mL each of three or four coded samples were presented in random order to the panelists who were asked to rank according to preference; one being most

preferred, and three or four (depending upon the number of samples) the least preferred. Data were tabulated and analyzed using a sign test (Steele and Torrie, 1960) on samples within each panel.

RESULTS & DISCUSSION

THE RESULTS of the taste panel rankings are presented in Fig. 1 and 2. Although few statistically significant preference differences were noted among treatments, those found were differences in which a lower TA or carbonation level was preferred over the highest level of that variable within the panel. Thus in Fig. 1, at a TA of 0.65%, while the two lower carbonation levels were equally preferred statistically, there was a significant preference for 3.0 volumes of gas over 3.5. Similarly, at a TA of 0.75%, the preference for the two lowest carbonation levels were not significantly different, but juice with 2.5 volumes of gas is preferred over that with 3.5. Lastly, in Fig. 2, at 3.0 volumes of gas, the lower TA juices did not differ in preference, but the 0.75% TA juice was preferred over the 0.95% TA sample.

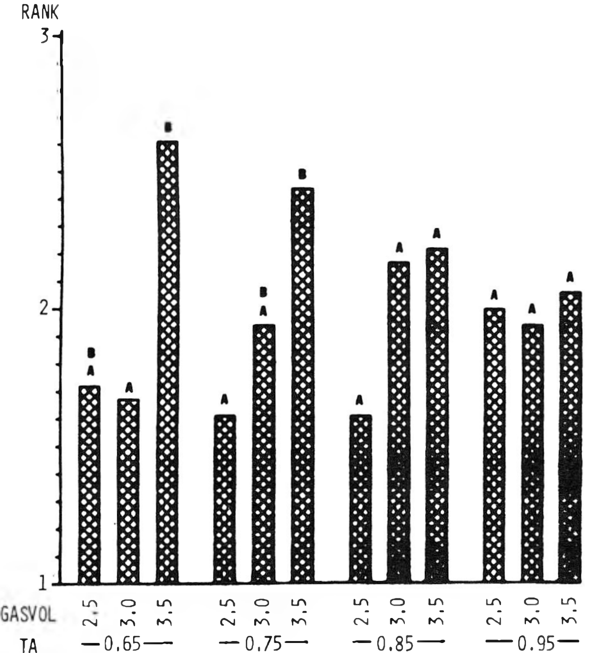

Fig. 2—Mean rank by taste panel of TA effect on preference of clarified carbonated pineapple juice at constant carbonation level. Within a gas volume, mean ranks are significantly different (P = 0.05) if they do not share a common letter.

Mean ranks for carbonation levels (Fig. 1) are 1.74, 1.93 and 2.33 for 2.5, 3.0 and 3.5 volumes of gas, respectively, indicating that overall the low level carbonation was preferred. This is consistent with general bottling practices: pineapple soda is generally charged with 2.5–3.0 volumes of carbon dioxide, while 2.0 volumes of gas are listed for a carbonated grapefruit juice based product (Jacobs, 1959).

Overall mean ranks for TA (Fig. 2) are 2.45, 2.26, 2.47, and 2.82 for 0.65%, 0.75%, 0.85% and 0.95%, respectively, indicating that a 0.75% TA product is preferred. On a Brix/acid ratio basis, a ratio of 18 was preferred, followed by ratios of 21 and 16, then lastly a ratio of 14. These results are comparable with those of Cavaletto (1981) who studied how dilution of pineapple juice, with subsequent adjustment of TA and °Brix, affected flavor perception. In a 50% juice drink adjusted to 14 °Brix, a Brix/acid ratio of 19 was preferred, followed by 24, which was slightly preferred over a ratio of 16. In a comparable set of samples adjusted to 12 °Brix, a ratio of 20 was preferred over 16, which was preferred over a ratio of 13.

Preference rankings do not give any information about consumer acceptability; however, most of the taste panelists found this product to be a pleasant drink. It is the belief of the authors that a sparkling clarified pineapple juice could be an acceptable and popular beverage, although the data herein suggest that a controlled composition would be required for optimal preference.

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THERMAL DIFFUSIVITY OF PAPAYA . . . From page 1219

$$\alpha = A + (\alpha_w - A)\,[\%\ \text{water (wt)}] \quad (7)$$

where $A = 0.833 \times 10^{-3}\ cm^2/s$ and α_w is the thermal diffusivity of water. The average value of α_w for this temperature range may be found from Bolz and Tuve (1973) to be $1.542 \times 10^{-3}\ cm^2/s$. Eq. (7) gives a value of $\alpha = 1.45 \times 10^{-3} \pm 0.01 \times 10^{-3}\ cm^2/s$ where the uncertainty value comes from the uncertainty in water content. Comparing this value of α with the experimentally determined values for pulp gives no significant difference using the student "t" test criterion.

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A Research Note

Evaluation of a Food Processor for Making Model Meat Emulsions

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ABSTRACT

Meat emulsions or batters made with a retail food processor were compared to batters produced by three commercial machines (silent cutter, Schnellkutter, and emulsifier) and two laboratory machines (small size cutter and blender) for suitability in laboratory studies. Batters were evaluated for post-heating fat losses and smokehouse water losses. Stabilities of the finished frankfurters were evaluated by severe cooking weight changes, penetration forces, fat droplet sizes, and sensory qualities. The food processor was comparable to the other machines for producing high quality batters and had the advantage of being inexpensive, economical with ingredients, and time-saving.

INTRODUCTION

MODEL SYSTEMS are frequently used to study meat emulsions. One approach has been to study the emulsifying capacity of meats by blending dilute protein extracts with liquid oil (Swift et al., 1961; Carpenter and Saffle, 1964; Webb et al., 1970). Although often used (Saffle, 1968; Webb, 1974), this approach has limited applicability in studying meat emulsions. Differences between this model system and actual meat emulsions include an unrealistically high emulsification capacity by the proteins in the model system, a much lower viscosity of the aqueous phase, the use of a liquid rather than solid lipid phase, and a lack of information on emulsion stability (Saffle, 1968).

Other model systems more closely simulate commercial procedures. Muscle tissue and vegetable oil were emulsified by a blender-mounted apparatus (Haq et al., 1972), and Ockerman and Cahill (1970) designed a small blender with a scraper in the bowl. Morrison et al. (1971) and Lauck (1975) used an Omni-Mixer. Johnson (1976) devised a prototype that incorporated a blender with a plunger to force the ingredients between the blades and through an exit tube. However, these methods were still not as close to commercial methods as desired, did not function well, or required special fabrication of the equipment.

The use of a food processor for making frankfurter batters has the potential for overcoming these objections. It can use the normal ingredients in the usual sequence of cutter steps. In addition, it has many potential advantages that include: being inexpensive, not requiring modification, and being capable of making many batches in a relatively short time. Therefore, we evaluated its batter-making capabilities compared to commercial and laboratory machines for use in meat research.

MATERIALS & METHODS

Materials

Beef round, pork shoulder, and pork adipose tissue were obtained from local processors. Proximate analyses were determined by the dye binding method for protein (Pettinati and Swift, 1976, personal communication) and the Foss-let method for fat (Pettinati and Swift, 1975). Frankfurters were formulated to contain 11% protein, 30% fat, 54% water, 2.51% salt, 1.98% sugar, 1.50% commercial spice mixture, 0.015% $NaNO_2$, and 0.053% ascorbate. The lean meats, salt, sugar, nitrite and ascorbate solutions, and ice were chopped for one-sixth to one-third of the total chopping time before fat was added. Batters were chopped to 15.5°C, stuffed into cellulose casings, and processed in an air conditioned smokehouse with wood smoke to an internal temperature of 71°C. Comparisons were made among the following machines (quantities and total chopping times): Cuisinart CFP-9 food processor (250g, 2 min); Koch-Alpina Pb-50 silent cutter (10 kg, 18.5 min); Stephen-Koch 25L Schnellkutter (7 kg, 5.8 min); Hobart-Stephan micro-cut MCU-12 emulsifier (7.5 kg, prechop 10.2 min in the Koch-Alpina silent cutter, then 1 pass through the emulsifier); Hobart 84145 small silent cutter (2.5 kg, 15.5 min); and Omni-Mixer Servall 485 laboratory blender (250g, 0.5 min). The laboratory blender was modified to allow the metal jar to be moved up and down relative to the blades for improved chopping of the entire sample (Morrison et al., 1971).

Analytical methods

Frankfurter quality was assessed by the fat loss during the emulsion stability test (Meyer et al., 1964), weight loss during processing, severe cook weight change (Tauber and Lloyd, 1947) after 10 min boiling, penetration force of a 6.4 mm plunger moving at 12.7 cm/min into the end of a 2.5 cm cylinder of frankfurter (Whiting et al., 1981), fat droplet size of 80 droplets (min size 25μ diameter) by direct microscopic examination of frankfurter sections stained with Sudan Black (Ackerman et al., 1971), and sensory triangle tests (Roessler et al., 1978; Whiting et al., 1981). The data were analyzed with a one-way analysis of variance and Duncan's multiple range test (Steel and Torrie, 1960).

RESULTS & DISCUSSION

OPERATION of the food processor was similar to the silent cutters. Lean meat was chopped with ice, salt and cure for 45 sec. After addition of fat, the mixture was chopped for an additional 30 sec and the temperature measured. The chopping and temperature measuring were repeated until the desired final temperature was reached. An emulsion could be chopped and the equipment cleaned for reuse in 15 min. Because of the high viscosity of the batters and long chopping times, the food processor should have protection against overheating the motor.

The food processor was capable of making emulsions of varying compositions (Table 1). Differences in smokehouse water losses, Meyer test fat losses, and penetration forces reflected the frankfurter's composition. The average fat droplet sizes and the slight weight gains when severely cooked show the processor's ability to handle reasonable variation. The 44.5% fat frankfurters showed a modest loss in the severe cook test. No fat separation occurred during smokehouse processing of any formulation.

Extending chopping times to reach higher temperatures produced unstable emulsions similar to that reported with larger cutter machines (Acton et al., 1983). Meyer test fat losses and severe cook losses indicated the loss of emulsifying capacity with increasing chopping temperature. Gel strength, as indicated by the penetration force, was slightly affected, and water loss decreased during the smokehouse processing. Although the average fat droplet size decreased,

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Table 1—Characteristics of batters and frankfurters made with a food processor

Treatment	Meyer test fat loss (%)	Smokehouse loss (%)	Severe cook wt change[d] (%)	Penetration force (g)	Average fat droplet size (μ)
Composition					
Low fat 15% protein, 11% fat	0.8[a]	16.8	1.6[c]	836[c]	103[b]
Standard 11% protein, 30% fat	2.0[b]	12.8	1.2[c]	518[b]	109[b]
High fat 9.5% protein, 37% fat	3.3[c]	10.6	0.0[b]	469[b]	106[b]
Very high fat 8% protein, 44.5% fat	2.7[bc]	7.0	-5.4[a]	322[a]	87[a]
Chopping temperature					
15.5°C	2.0[a]	12.8	1.2[a]	518[a]	109[a]
24°C	5.2[b]	11.0	-15.6[b]	455[b]	110[a]
31°C	14.0[c]	7.0	-46.8[c]	440[b]	94[b]

[a-c] Within an experiment, values in each column with the same letters are not significantly different ($p < 0.05$).
[d] Positive values indicate a weight gain, negative values, a weight loss.

Table 2—Comparison of emulsions and frankfurters made by the food processor and commercial or laboratory machines

Machine	Meyer test fat loss (%)	Smokehouse shrinkage (%)	Severe cook wt change[e] (%)	Penetration force (g)	Average fat droplet size (μ)	Triangle test with food processor
Food processor	0.8[a]	11.0	1.2[bcd]	424[a]	120[c]	
Silent cutter	0.7[a]	10.6	-1.4[a]	486[b]	73[a]	26/44***
Schnellkutter	3.3[c]	10.9	1.4[cd]	453[ab]	70[a]	13/24*
Emulsifier	2.0[abc]	10.8	0.4[bc]	450[ab]	83[ab]	14/22**
Small silent cutter	2.6[bc]	11.0	0.3[b]	602[c]	113[c]	–
Laboratory blender	1.5[ab]	12.0	1.7[d]	558[c]	104[bc]	–

[a-d] Values in each column with the same letter are not significantly different ($p < 0.05$).
[e] Positive values indicate a weight gain, negative values a weight loss.
* $p < 0.03$; ** $p < 0.01$; *** $p < 0.001$.

more droplets larger than 200 μ were observed in sausages from the 31°C treatment which Ackerman et al. (1971) showed was indicative of emulsion failure. Extensive fat caps were also observed.

Preparations made in three commercial machines (silent cutter, Schnellkutter, and emulsifier) and two laboratory machines (small silent cutter and blender) were compared with those made in the food processor (Table 2). All batters were of standard composition (11% protein, 30% fat), chopped to 15.5°C, and processed in the smokehouse at the same time. Smokehouse shrinkages of the batters were all similar. The fat losses and severe cook changes showed that a good quality batter could be produced by each machine. Penetration force was lowest and fat droplets largest for the frankfurters made with the food processor, but the values were not significantly different than those from some of the other machines. Connective tissue was visible in the emulsions chopped with the small silent cutter and the laboratory blender and probably caused their high penetration forces. The three laboratory machines had larger fat droplets including a few fat droplets larger than 200 μ in diameter. However, no fat separation was observed in any of the frankfurters.

The triangle tests indicated significant differences in sensory quality among frankfurters made with the food processor and the three commercial type machines, although the portion of correct judgments ranged from only 13 of 24 judgments to 14 of 22 judgments. This demonstrated that even though the franks were not identical, the differences were not of a magnitude that was consistently detected.

In summary, these tests showed that differences existed between batters manufactured by any of the machines. The food processor made a high quality batter and had many advantages for laboratory use.

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A Research Note

Use of Glandless Cottonseed and Sunflower Seed Products in Batter/Breading for Fried Beef Patties

KI SOON RHEE

ABSTRACT

This study indicates that defatted glandless cottonseed flour (GCF) or sunflower seed protein isolate (SI) incorporated in the batter/breading system can impart their antioxidant effect to the meat of fried beef patties. Coarsely ground lean beef patties were coated consecutively with dry ingredient (wheat flour for control and 1:1 mixture of wheat flour-GCF or wheat flour-SI for experimental samples), batter made of 1 part of dry ingredient and 2 parts of water, and finally dry ingredient. Ground beef and dry ingredients each contained 1% salt. Use of GCF or SI in the coating mixes greatly reduced the thiobarbituric acid values of the meat when the coated patties were fried and stored at 4°C for 5 days.

INTRODUCTION

THE DEVELOPMENT of rancidity caused by lipid peroxidation is the primary quality deterioration problem in uncured, precooked or cooked meat products. Control of rancidity development in these products has become more critical with the increased use of precooked meat items by the foodservice industry. Batter-fried or breaded fish sticks and patties and chicken nuggets are popular precooked or fast foodservice entree items that have been widely marketed in recent years. Following the success of these products in the market, some meat companies have developed breaded, precooked chicken patties and breaded, batter-fried beef patties (often called "chicken-fried" beef patties or steaks). Many of these products, as marketed at present, contain soy ingredients and phosphates in the meat. Rancid and other off-flavors are a serious problem in these precooked meat products as currently marketed.

Ziprin et al. (1981) reported that, when food ingredients prepared from soy, peanut and glandless cottonseed were incorporated in raw ground beef, these oilseed products provided antioxidant protection to refrigerated, cooked ground beef patties, with the highest antioxidant potential shown by the cottonseed products. Use of these oilseed products in gravy was also shown to reduce lipid peroxidation in refrigerated, cooked ground beef patties that were covered with the gravy (Rhee and Ziprin, 1981). The identity of antioxidant compounds in cottonseed products is not clearly known; however, it is likely to be polyphenolic compounds. Antioxidant properties of soybeans and soybean products are due primarily to flavonoids and phenolic acids (Pratt and Birac, 1979) and cottonseed products are known to contain various flavonoids and phenolic acids (Blouin et al., 1981; Cairns, 1978; Sosulski, 1979b). Sunflower seed products are also likely to possess antioxidant characteristics since they contain large concentrations of polyphenolic compounds (Cater et al., 1972; Sosulski, 1979a). The current study was undertaken to determine whether defatted cottonseed flour (GCF) or sunflower seed protein isolate (SI) used in the batter/breading system can impart their antioxidant effect to the meat of fried beef patties.

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MATERIALS & METHODS

FRESH BEEF TOP ROUND purchased from a local supermarket was trimmed of all visible external fat, ground once through a plate with 9.53-mm holes, and divided into two batches. One batch of ground beef was immediately frozen at −20°C. The oilseed products, GCF and SI, were obtained from the Food Protein Research & Development Center, Texas A&M Univ. They were prepared by hexane extraction of the oilseeds and alkali extraction and isoelectric precipitation of the proteins. The color of GCF was slightly yellow and that of SI was dark and greenish.

In Experiment 1, fresh ground beef (the unfrozen batch) was mixed with 1% salt (percentage based on meat weight) and formed into patties (80g each, 9.5 cm diameter). Each patty was coated consecutively with 5g dry ingredient (wheat flour for control and 1:1 mixtures of wheat flour-GCF or wheat flour-SI for experimental samples) containing 1% salt, 30g batter (1 part dry ingredient containing 1% salt plus 2 parts water), and finally 14g dry ingredient with 1% salt. Two patties of the same batter/breading treatment at a time were fried (3 min per side) in an electric skillet at 176.7°C using 120 mL commercial cooking oil (partially hydrogenated soybean oil). Fresh cooking oil was used each time. After frying, patties were placed on paper towels for approximately 3 min to drain excess oil, placed in Whirl-Pak bags (O_2 permeability, 12,400 $cc/m^2/24$ hr), and stored at 4°C for 5 days.

In Experiment 2, the frozen (6 days at −20°C) batch of ground beef was thawed at 4°C overnight, mixed with 1% salt, and formed into patties (70g each, 9.1 cm diameter). Salt was also added to dry coating ingredients at 1% levels. Each patty was first coated with a dry coating ingredient (wheat flour or 1:1 mixture of wheat flour-oilseed ingredient), shaking off any excess dry ingredient, and then with batter (1 part dry coating ingredient + 2 parts water). The batter-coated patties were placed on a rack for approximately 3 min to allow excess batter to drain. Finally, patties were coated again with a dry ingredient – as much as would adhere to each patty. Patties were then fried and stored as described for Experiment 1.

The extent of lipid peroxidation was determined by the thiobarbituric acid (TBA) test as modified by Rhee (1978). Color was developed in duplicate for each distillate. Where feasible, data were analyzed by analysis of variance and mean separation by the Student-Neuman-Keuls' test (Steel and Torrie, 1980).

RESULTS & DISCUSSION

RESULTS of Experiments 1 and 2 are shown in Table 1. Incorporation of GCF or SI into the batter/breading system (crust mix) greatly reduced TBA values of the meat of fried beef patties that were stored for 5 days at 4°C, with little difference found between GCF and SI in antioxidant effectiveness. For each crust ingredient treatment, initial (0-day) TBA values of the meat were higher in Experiment 2 than in Experiment 1, probably because frozen-thawed ground beef was used in Experiment 2, as indicated previously. Freeze-thawing of meat is known to accelerate lipid peroxidation (Ledward and Macfarlane, 1971). TBA values of crusts (formed from dry coatings plus batter on frying) of different compositions were low and not discernibly different from each other. TBA values of whole fried patties (crust + meat) were determined only in Experiment 1; the values were 7.2, 1.8 and 2.5 for the crust treatments of wheat flour alone, GCF plus wheat flour and SI plus wheat flour, respectively.

It is not known whether the antioxidant protection provided to the meat by GCF or SI in the crust mix is through

Table 1—TBA values for fried beef patties[a] stored at 4°C for 5 days

Ingredient in crust mix[b]	TBA value[c] (mg malonaldehyde/kg sample)			
	Crust		Meat	
	0 day	5 days	0 day	5 days
Experiment 1[d]				
Wheat flour (100%)	0.9	1.3	1.0	10.9
GCF + wheat flour (50% + 50%)	0.6	0.7	1.1	2.7
SI + wheat flour (50% + 50%)	0.7	0.9	1.4	3.7
Experiment 2[e]				
Wheat flour (100%)	1.3	1.7[f]	3.6	10.5[f]
GCF + wheat flour (50% + 50%)	1.3	1.5[f]	3.0	4.3[g]
SI + wheat flour (50% + 50%)	1.6	1.6[f]	3.4	4.2[g]

[a] Before frying, each beef patty was coated consecutively with dry ingredient (breading), batter (1 part dry ingredient + 2 parts water), and finally dry ingredient.
[b] GCF, defatted glandless cottonseed flour; SI, sunflower seed protein isolate.
[c] TBA values were analyzed statistically only for those crust and meat samples of 5-day storage in Experiment 2, because of lack of patty replication for other data sets.
[d] For each storage period, one patty was used to obtain the crust and meat values.
[e] For 0-day storage period, one patty was used for the crust and meat values; three patties were used for 5-day storage period.
[f,g] Means within a column within the same experiment which do not bear a common superscript are significantly different ($P < 0.05$).

migration of antioxidant substances originating from GCF or SI to the meat during and/or after frying or through a surface phenomenon. This question needs to be addressed in future research.

Cursory observations (not quantitated) of the fried products indicated that addition of GCF also improved appearance and texture of the crust, over use of wheat flour alone, with more golden brown appearance and crispier texture. In contrast, use of SI discolored the crust due to the dark greenish color of SI. The color problem for sunflower seed products is caused primarily by the oxidation of chlorogenic acid abundantly present in this seed (Sosulski, 1979a), the problem being most serious for protein isolate prepared by alkali extraction. However, a white-colored SI prepared by a new process (O_2 expulsion and exclusion process) developed by Lawhon et al. (1982) may be used in the batter/breading system without affecting the crust color acceptability. Further research is needed to determine the qualitative and quantitative effects of using various antioxidant oilseed food ingredients in batter-coated/breaded, fried meat products.

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A Research Note

Consumer Acceptance of Prerigor Pressurized Versus Conventionally Processed Utility Grade Beef

Z. A. HOLMES

ABSTRACT

Consumer tests of institutional prerigor pressurized and conventionally aged shoulder clods and/or inner chucks from utility grade cows were tested in a local soup kitchen, college women's cooperative living group and a university catering or banquet food service. The 318 member panels evaluated the prerigor pressurized beef as being more acceptable than conventionally processed beef. Differences in degree of acceptability at each location may be due to method of preparation.

INTRODUCTION

PRERIGOR PRESSURIZATION (PRP) of beef has been reported to have a beneficial effect on quality characteristics. Macfarlane (1973) indicated prerigor pressurization of longissimus dorsi muscle of beef improved tenderness and juiciness panel scores. Similar results have been reported by Kennick et al. (1980) and Riffero and Holmes (1983). Koohmaraie et al. (1983) noted improved color of PRP meat. With a trained sensory panel (Riffero and Holmes, 1983), PRP semitendinosus was evaluated to be significantly more tender, with easier fiber separation than conventionally processed beef. The purpose of this reported research was to compare the influence of conventionally processed versus prerigor pressurized processed beef on consumer acceptability.

MATERIALS & METHODS

EIGHT HEAD of utility grade cows (353 - 538 kg live wt) were conventionally slaughtered at the Clark Meat Science Laboratory, Oregon State Univ. Shoulder clod and/or inner chuck roasts from one side of each carcass were randomly assigned to the conventionally processed control and those from the other side to prerigor pressurization. Beef for PRP was vacuum packed in Cry-O-Vac bags, inserted into a pressure chamber (30.5 × 60.96 cm) and a pressure of 103.5 MNm^{-2} (15,000 lb/sq in) applied for 2 min and frozen. The matching muscles on the opposite side were left on the carcasses and chilled at 0 ± 1°C according to commercial practices. On the 7th day the matching muscles from the CONV processed sides were removed from the carcasses and vacuum packed. Upon completion of processing, the eight paired vacuum-packed shoulder clods (6.0 - 7.9 kg) and four paired inner chucks (3.2 - 5.8 kg) were frozen and stored (−18°C) until used.

Prior to use, frozen roasts were defrosted (5°C) for 36 hr. Shoulder clods or inner chuck cuts were roasted and then cooled to 30 - 40°C for Experiment I and II. Lean portions of each cut were sliced approximately 1/4 in, warmed to serving temperature and used for sensory evaluation. For each experiment all beef was served as part of a meal. The consumer received either CONV or PRP as coded by the investigator. The scorecard shown in Fig. 1 was used for the three different consumer groups. The number of panelists for each CONV versus PRP processing comparison, the number of panelists scoring each acceptability rating within a treatment was converted to a percentage value by dividing by the total panelists evaluating each treatment. Chi-square tests ($P \leq 0.05$) were done using these percentage values with the Statistics 3.0 (Edu-Ware Services, Inc. Agoura, CA 91301) statistical package for the APPLE IIe.

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Experiment I

A paired shoulder clod or inner chuck of PRP and conventionally processed beef was roasted (163 ± 15°C) in an electric oven to an endpoint temperature of 70°C. The lean of the cooked beef was sliced and served on Wednesday at a local free meal facility, Stone Soup, St. Mary's Catholic Church. Paired PRP and conventionally processed shoulder clods and inner chucks were evaluated at three and four different meal periods, respectively. The 183 panelists were 63% male and 32% female and ranged in age from under 15 to over 60 with 71% being 16 - 45 years old.

Experiment II

Paired shoulder clods of PRP and conventionally processed beef were evaluated at Azalea Cooperative, Oregon State Univ. Each beef cut was roasted in an electric oven (177 ± 10°C) to an endpoint temperature of 70 - 75°C. All panelists were females 16 - 30 years old.

Experiment III

Lean meat from two paired inner chucks of PRP and conventionally processed beef was excised and used in two batches of beef burgundy for the annual home economics alumni dinner catered by the university food service. The beef-sauce mixture was served on top of noodles. The 64 consumer panelists were 75% female with approximately 60% of the panelists being 31 - 60 years of age.

RESULTS & DISCUSSION

AN EVALUATION of the data (Table 1) from the consumer panels indicates the PRP processed meat was generally more acceptable than the conventionally processed beef. Although this greater acceptability of PRP beef was shown for each group, the degree of difference varied with all but the inner chuck comparisons in Experiment I being significantly ($P \leq 0.05$) different. The women in the cooperative (Experiment II) showed the greatest ($P \leq 0.05$) difference between CONV and PRP processed beef in the regions of greatest acceptability. This is possibly due to the higher oven temperature used with the utility grade shoul-

ACCEPTANCE OF THE BEEF

☐ ☐ ☐ ☐ ☐

PLEASE CHECK THE BOX UNDER THE FIGURE WHICH BEST DESCRIBES HOW YOU FEEL ABOUT THE BEEF YOU ATE! ADDITIONALLY, I WOULD APPRECIATE YOUR FURNISHING THE FOLLOWING OPTIONAL INFORMATION.

PLEASE CHECK.

___ MALE
___ FEMALE

APPROXIMATE AGE

___ 0 - 15 YEARS
___ 16 - 30 YEARS
___ 31 - 45 YEARS
___ 46 - 60 YEARS
___ 61 - 110 YEARS

Nº 0501

Fig. 1—Scorecard used by consumers to record evaluation of prerigor pressurized or conventionally processed beef.

Table 1—Consumer acceptability of prerigor pressurized versus conventionally processed utility shoulder clod and inner chuck beef[a,b]

	EXPERIMENT I				EXPERIMENT II		EXPERIMENT III	
	Shoulder clod*		Inner chuck		Shoulder clod*		Shoulder clod*	
	CONV	PRP	CONV	PRP	CONV	PRP	CONV	PRP
Number of panelists	42	46	46	49	36	35	30	34
Acceptability								
	33.2	37.0	39.1	42.9	5.6	31.4	30.0	20.6
	45.2	30.4	28.3	24.5	41.7	45.7	36.7	50.0
	16.7	30.4	23.9	26.5	44.4	17.1	10.0	17.6
	2.4	2.2	8.7	4.1	5.6	5.7	16.7	5.9
	2.4	0.0	0.0	2.0	2.8	0	6.7	5.9

[a] Conventionally (CONV) and prerigor pressurized (PRP) processed beef.
[b] Percent response calculated from number of panelists for each acceptability rating divided by total panelists for each CONV or PRP processing treatment.
Chi square comparison of CONV to PRP, *P ≤ 0.05

der clod. Although processing significantly (P ≤ 0.05) affected acceptability in Experiment III, the panelists reported CONV processed beef being most acceptable at the highest acceptability level. This difference from Experiment I and II could be due to both meats being prepared by a braising method. However, in all three experiments, panelists evaluated PRP beef more acceptable than CONV beef when the highest three levels of acceptability were grouped.

The greater acceptability of prerigor pressurized meat in consumer tests of the current experiment would further substantiate the advantages of PRP for low quality beef. In a number of studies (Kennick et al., 1980; Pratt, 1977; Macfarlane, 1973; Riffero and Holmes, 1983) utilizing trained panels, prerigor pressurized beef was observed to be significantly more tender. A number of other studies evaluating production and processing also indicated the advantages of PRP processing. Schumann et al. (1982) observed prerigor pressurized beef to have a higher yield in total wholesale cuts than conventionally processed beef. In a comparison of PRP versus CONV processed retail cuts (Koohmaraie et al., 1983), PRP beef steaks were observed to have a significantly (P ≤ 0.05) better color and less discoloration at the end of 5 days in a fluorescent lighted retail case. Thus, prerigor pressurization of beef appears to offer potential advantages from both the production and consumption viewpoints.

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

A Research Note

Starch Digestibility as Affected by Polyphenols and Phytic Acid

LILIAN U. THOMPSON and JANE H. YOON

ABSTRACT

The rate of wheat starch digestibility in the presence or absence of polyphenols (catechin or tannic acid) and/or phytic acid at concentrations found in legumes was determined in an *in vitro* dialysis system. Addition of tannic acid and phytic acid reduced the starch digestibility 13 and 60% respectively, at 5 hr. Combined tannic and phytic acid reduced the digestibility at a level (63%) which did not differ significantly from that with only phytic acid. Catechin had no significant effect on rate of starch digestibility.

INTRODUCTION

LEGUMES have been successfully used for the better management of diseases such as diabetes and hyperlipidemia (Anderson and Kyllen, 1979; Jenkins, 1982; Jenkins et al., 1983a, b; Kiehm et al., 1976; Miranda and Horwitz, 1978; Rivellese et al., 1980; Simpson et al., 1981). This appears to relate to the rate at which legumes release their products of starch digestion (Jenkins et al., 1982b). Legumes which release glucose at a slower rate than cereals both *in vivo* (Jenkins et al., 1980; Potter et al., 1981; Dilawari et al., 1981) and *in vitro* (Jenkins et al., 1980; Jenkins et al., 1982c) are good sources of fiber and antinutrients (Liener, 1980). Although dietary fiber among many other factors (Thorne et al., 1983) has received much attention to explain the slow rate of carbohydrate digestion, the role of antinutrients has recently been questioned.

A strong negative relationship has been observed between polyphenol intake and blood glucose response of normal and diabetic individuals (Thompson et al., 1984) suggesting an effect of polyphenols on rate of carbohydrate digestion. Since other antinutrients such as phytic acid also negatively correlated with blood glucose response (Yoon et al., 1983) and since they also exist in foods rich in polyphenols such as legumes, the sole effect of polyphenols on the rate of starch digestion was investigated in this study. Polyphenols in the form of catechin or tannic acid were added to wheat starch and the rate of starch digestibility observed in an *in vitro* dialysis system (Jenkins et al., 1982c). The effect of phytic acid in the presence or absence of polyphenols was also examined.

MATERIALS & METHODS

TO 10 mL FRESH POOLED human saliva was added 4.83 mg tannic acid (with 9% moisture, BDH Chemicals Ltd. (Poole, England) and equivalent to 4.4 mg polyphenols) or 5.35 mg catechin (with 3.5 H_2O/mole, Sigma Chemical Co., and equivalent to 4.4 mg polyphenols) and incubated at 37°C for 30 min. The pH was adjusted to 7.0 before and after the incubation. Raw wheat starch (Charles Tennant and Co., Ltd.) (2g) was then added and the mixture was made up to 35 mL with distilled water, placed in a dialysis bag (12,000 MW cut off) and dialyzed against 800 mL distilled water at 37°C. For 5 hr, hourly samples (5 mL) were taken from the dialysate and analyzed for starch digestion products (glucose, maltose, maltotriose) by high pressure liquid chromatography (Waters Ltd. HPLC system, WISP automatic injector, Model 6000A pump, radial compression module, Dextropac column with water as a mobile phase at 1.5 mL/min and differential refractometer detector).

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The same dialysis system was employed in determining the effect of 66 mg sodium phytate (containing 12% moisture, Sigma Chemical Co. and equivalent to 40 mg phytic acid) alone or together with 4.83 mg tannic acid or 5.35 mg catechin. The phytic acid with or without polyphenols and saliva was preincubated at 37°C for 30 min prior to mixing with the starch. The pH was also adjusted to 7.0 before and after incubation.

In all cases, wheat starch with no added antinutrients served as a control. Each test was done in duplicate. Data at each digestion time were analyzed by one way analysis of variance and the significance of difference was determined by Tukey's Test (Snedecor, 1956).

RESULTS & DISCUSSION

IN THIS STUDY, *in vitro* dialysis system was employed since the sugars released from the dialysis bag has been shown to relate signficantly to blood glucose response (Jenkins et al., 1982a). The use of saliva as source of amylase was justified by its ready availability and similarity in starch digestibility to results obtained with human pancreatic juices (Jenkins et al., 1982c). Polyphenols and phytic acid were added to the wheat starch to make their concentration approximately equal to that of the legumes i.e. 0.22% and 2% respectively, based on starch portion (Thompson et al., 1984; Yoon et al., 1983) since legumes produced lower blood glucose responses compared with cereals. Wheat starch was used since it has negligible amounts of polyphenols and phytic acid compared with legume starch preparations which have small amounts of these substances (unpublished data) which may complicate the experiment.

Polyphenols and phytic acid may affect starch digestibility through interaction with amylase enzyme, with protein which is closely associated with starch or directly with the starch (Thompson et al., 1984; Yoon et al., 1983; Deshpande and Salunke, 1982; Tamir and Alumot, 1969). In addition, in the case of phytic acid, the effect may be due to binding with calcium which is known to catalyze amylase activity (Yoon et al., 1983). Preincubation of the antinutrients with the enzyme prior to addition of starch was done in this study to enhance any effect on digestibility due to interaction with the amylase enzyme.

The total sugars released in the dialysate over a 5-hr period is given in Fig. 1. The distribution of glucose, maltose and maltotriose in the total sugar released from wheat starch at a given digestion time was the same regardless of whether it was digested with or without the presence of phytic acid and/or polyphenols (Table 1). For all samples, maltose was the major product released followed by maltotriose and glucose. With longer digestion times, the proportion of maltotriose released decreased while that of maltose and glucose increased.

Tannic acid significantly reduced the starch digestibility of the control by 13% ($p < 0.05$) at 5 hr while catechin had no significant effect (Fig. 1). This suggests the greater reactivity of tannic acid than catechin and the variability between polyphenols in terms of effect on starch digestibility.

The addition of phytic acid significantly reduced the starch digestibility by 28% ($p < 0.05$) at 1 hr and 60% ($p < 0.05$) at 5 hr. When both catechin and phytic acid were added to the starch, the digestibility was similar to that seen with only phytic acid added.

The addition of both tannic acid and phytic acid lowered the digestibility of starch by 63% at 5 hr but this did not differ sig-

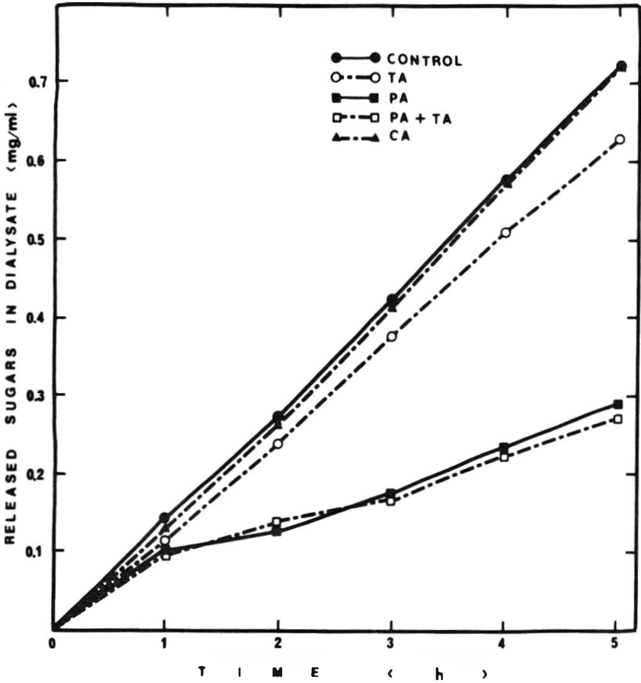

Fig. 1—Rate of starch digestibility in the presence or absence of tannic acid (TA), catechin (CA) and/or phytic acid (PA) at pH 7.0.

Table 1—Percent of glucose, maltose and maltotriose in the total sugars released from wheat starch samples digested in the presence or absence of polyphenols and/or phytic acid

Time (hr)	Glucose	Maltose	Maltotriose
1	4.6	63.2	32.2
2	5.3	66.0	28.7
3	7.1	67.6	25.3
4	7.4	69.4	23.2
5	8.3	70.4	21.3

nificantly from the 60% observed with phytic acid alone. Therefore, there was no additive or synergistic effect between these two antinutrients. Probably a maximum reduction in starch digestibility has been attained with the level of phytic acid added to the starch and that further addition of tannic acid or catechin does not produce additional effect; or since the reduction on starch digestibility by polyphenol was small, it may be obscured by the large reduction by phytic acid. A future dose-response study of these antinutrients and starch digestibility may be helpful in further understanding the interaction between phytate and polyphenol.

In conclusion, polyphenols such as tannic acid play a role although a smaller role compared to phytic acid in reducing the *in vitro* rate of starch digestibility and possibly blood glucose response. Different polyphenols influence the rate of digestibility at varying degrees.

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A Research Note

Freezing Time Predictions for Different Final Product Temperatures

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ABSTRACT

A modification to an empirical food freezing time prediction formula is proposed that allows the formula to be used for a range of final product temperatures. Over a large data set (275 runs) the percentage difference between experimentally measured times and predictions has a mean of 0.2% and a standard deviation of 6.8% for the improved formula compared to −2.4% and 8.5%, respectively, prior to modification. Ninety percent of the predicted freezing times by the new method were within ±11% of the experimentally measured times and ±9% of predictions by an accurate finite difference scheme. This performance compares favorably with other published freezing time prediction methods. The same type of modification for varying final product temperature may be suitable for other empirical formulae.

INTRODUCTION

ONE OF THE DESIRED consequences arising from the use of a recently proposed systematic methodology for assessment of freezing time prediction methods for foods (Cleland and Earle, 1984) was that areas in which improvements to prediction methods could be made would emerge. One area highlighted was that prediction formulae based on curve-fitting of experimental data collected in experiments terminated at one final product temperature performed poorly in comparison when applied to situations with different final product temperatures. This was because there is no term for varying T_{fin} in such prediction formulae. The formulae of Cleland and Earle (1982) and Hung and Thompson (1983) were found to be limited in this way, whereas those of Pham (1984a, b) and de Michelis and Calvelo (1983) were not. This note proposes a modification to the Cleland and Earle formula of the form:

$$t_f = (t_f \text{ to } T_{ref}) \cdot \left[1 - \frac{1{\cdot}65\ Ste}{k_s} \ln\left(\frac{T_{fin} - T_a}{T_{ref} - T_a}\right)\right] \quad (1)$$

where T_{ref} is −10°C, and T_a must be less than T_{ref}. A further condition is that T_{fin} must be below the temperature range where the bulk of the latent heat release takes place. The modification may also be applicable to other empirical formulae such as that of Hung and Thompson.

The form of Eq. (1) was derived by consideration of finite difference results in Cleland and Earle (1984). Plots of $\ln[(T_{fin} - T_a)/(T_{ref} - T_a)]$ versus time were constructed using temperature data after the main phase change region. As would be expected from heat conduction theory for constant thermal properties these showed straight lines. The slopes of the straight lines were linearly related to the Stefan number, and were found to be inversely proportional to the frozen material thermal conductivity. The factor of 1.65 was the fitted constant required. This constant applies for all materials. The variation of it between materials, and between different Biot numbers was sufficiently small (less than 10%) for a single average value to be used.

RESULTS

THE SPECIFIC FORM of the new Cleland and Earle formula uses T_{ref} = −10°C (because −10°C was the final product center temperature in the data set from the earlier work) and is:

$$t_f = \frac{\Delta H_{10}}{(T_f - T_a)\,(EHTD)}\left(P\frac{D}{h} + R\frac{D^2}{k_s}\right)\left[1 - \frac{1.65\ Ste}{k_s}\ln\left(\frac{T_{fin} - T_a}{-10 - T_a}\right)\right] \quad (2)$$

$$\text{where } P = 0.5\,[1.026 + 0.5808\ Pk + Ste\,(0.2296\ Pk + 0.1050)] \quad (3)$$

$$R = 0.125\,[1.202 + Ste\,(3.41\ Pk + 0.7336)] \quad (4)$$

$$Pk = C_L(T_i - T_f)/\Delta H_{10} \quad (5)$$

$$Ste = C_s(T_f - T_a)/\Delta H_{10} \quad (6)$$

and EHTD is defined in the manner of Cleland and Earle (1982).

As a calculation example the problem in Heldman (1983) was chosen. A 2 cm diameter strawberry is frozen from 10°C initial temperature to a final temperature of −25°C. The cooling medium is at −35°C, and the surface heat transfer coefficient is 70 W/m²°C. Thermal data were taken from Table 3 of Cleland and Earle (1984), using the procedures described in that paper to evaluate frozen phase properties:

$C_L = 4.09 \times 10^6$ J/m³°C $C_s = 2.15 \times 10^6$ J/m³°C
$\Delta H_{10} = 318 \times 10^6$ J/m³ $k_s = 2.08$ W/m°C
$T_f = -0.5$°C

Application of Eq. (2) to (7) led to the following parameters:

$T_a = -35$°C $T_{fin} = -25$°C
$T_i = 10$°C $EHTD = 3$
$Pk = 0.135$ $Ste = 0.233$
$P = 0.568$ $R = 0.185$

$(T_{fin} - T_a)/(-10 - T_a) = 0.400$; $t_f = 10.13 \times 1.169$ min = 11.85 min.

This freezing time to −25°C of 11.85 min is close to the finite difference prediction by Scheme A in Cleland and Earle (1984) which was 11.55 min. The freezing time to −10°C is 10.13 min by this procedure. These results appear plausible but testing is required over a range of conditions so new calculations were carried out for the composite data set in Cleland and Earle (1984). The consequential changes to Tables 6 and 8 of that paper are shown in Tables 1 and 2 respectively.

It was also considered worthwhile to attempt to apply the analogous procedure to modify the method of Hung and Thompson (1983) using T_{ref} = −18°C.

$$t_f = \frac{\Delta H_{18}}{U(T_f - T_a)(EHTD)}\left(P\frac{D}{h} + R\frac{D^2}{k_s}\right)\left[1 - \frac{1.65\ Ste}{k_s}\ln\left(\frac{T_{fin} - T_a}{-18 - T_a}\right)\right] \quad (8)$$

$$\text{where } P = 0.7306 - 1.083\ Pk + Ste\,(15.40U - 15.43 + 0.01329\ Ste/Bi) \quad (8)$$

$$R = 0.2079 - 0.2656\ U\ Ste \quad (9)$$

$$Pk = C_L(T_i - T_f)/\Delta H_{18} \quad (10)$$

$$Ste = C_s(T_f - T_a)/\Delta H_{18} \quad (11)$$

$$U = 1 + \frac{0.5C_L\,(T_i - T_f)^2 - 0.5C_s\,(T_f + 18)^2}{\Delta H_{18}\,(T_f - T_a)} \quad (12)$$

$$\text{and } Bi = hD/k_s \quad (13)$$

This procedure can only apply for values of T_a below −18°C.

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Table 1—Summary of comparisons between experimental and predicted freezing times for five prediction methods[a]

	Methods									
	Finite differences		Cleland and Earle		Pham		Hung and Thompson		de Michelis and Calvelo	
Data source	Mean	s.d.	Mean	s.d.	Mean	s.d.	Mean	s.d.	Mean	s.d.
Hung and Thompson										
23 Tylose slabs	−1.3	8.7	−3.3	7.0	−3.8	7.7	1.1	2.9	−4.3	14.1
9 potato slabs	−3.9	4.9	−7.3	3.3	−5.1	3.5	2.9	4.0	−4.2	8.4
9 carp slabs	3.1	12.9	−1.6	9.9	−2.4	11.1	2.8	4.1	6.1	16.9
9 ground beef slabs	6.6	16.6	2.6	13.4	−1.8	13.9	2.5	4.8	10.1	18.9
9 lean beef slabs	2.8	11.6	−4.3	7.4	−6.0	8.9	0.8	4.0	7.9	14.7
Cleland and Earle										
43 Tylose slabs	0.0	5.3	1.6	2.8	3.9	4.4	8.0	11.9	−4.1	6.2
6 potato slabs	−0.5	5.1	−0.5	1.8	3.7	2.3	11.3	5.7	−2.6	3.5
6 lean beef slabs	4.8	4.7	2.1	4.0	4.1	4.6	10.8	11.8	5.6	4.4
30 Tylose cylinders	−1.8	5.2	−1.0	3.6	0.8	5.1	1.8	7.9	−6.5	8.2
30 Tylose spheres	−0.3	3.3	1.6	5.2	2.3	5.1	3.5	8.6	−0.5	10.3
72 Tylose bricks	−3.8	5.8	−0.9	5.7	−1.3	4.7	0.8	9.5	−26.1	9.6
de Michelis and Calvelo										
5 lean beef slabs	−0.1	7.4	8.2	2.6	−0.9	4.9	1.8	9.2	−2.2	10.5
24 lean beef bricks, rods, finite cylinders	8.6	6.3	8.2	10.0	4.1	6.6	3.8*	11.5*	−11.3	10.8

a All data expressed as percentage differences from experimental data. Results for the Cleland and Earle, and Hung and Thompson methods were calculated by the modified forms.
* Ignores 4 experiments in which $T_a > T_{ref}$

Table 2—Summary of percentage differences between (A) experimental data and predicted freezing times, and (B) predicted freezing times from the methods under test and predicted freezing times from finite differences[a]

	Methods			
Data source	Cleland and Earle	Pham	Hung and Thompson	de Michelis and Calvelo
All 275 experiments; (A) comparison to experimental data				
Mean	0.2	0.3	3.2*	−8.7
Standard deviation	6.8	6.7	9.3	15.1
Maximum	23	18	33	38
Minimum	−21	−24	−18	−48
Range enclosing 90% of data	−10 to 11	−10 to 9	−11 to 20	−35 to 15
Correlation (r) with finite differences	0.746	0.762	0.460	0.619
207 experiments to −10°C; (A) comparison to experimental data				
Mean	0.5	1.3	3.5*	−11.8
Standard deviation	6.0	5.4	10.1	13.9
Maximum	23	15	33	16
Minimum	−21	−13	−18	−48
Range enclosing 90% of data	−9 to 10	−9 to 8	−12 to 21	−36 to 8
Correlation (r) with finite differences	0.665	0.738	0.522	0.501
68 experiments to −18°C; (A) comparison to experimental data				
Mean	−1.1	−2.8	2.3	0.8
Standard deviation	9.4	9.2	6.2	14.9
Maximum	22	18	30	38
Minimum	−21	−24	−6	−28
Range enclosing 90% of data	−13 to 15	−17 to 13	−5 to 9	−17 to 28
Correlation (r) with finite differences	0.838	0.929	0.552	0.857
All 275 experiments; (B) comparison to finite differences				
Mean	−0.5	0.5	3.0*	−8.4
Standard deviation	5.3	5.1	8.2	12.0
Maximum	17	14	31	15
Minimum	−18	−15	−19	−50
Range enclosing 90% of data	−9 to 8	−9 to 8	−8 to 17	−30 to 9
207 experiments to −10°C; (B) comparison to finite differences				
Mean	1.3	2.1	4.4*	−11.0
Standard deviation	5.0	4.3	8.7	12.1
Maximum	18	14	31	15
Minimum	−21	−14	−21	−49
Range enclosing 90% of data	−7 to 9	−5 to 8	−9 to 19	−31 to 6
68 experiments to −18°C; (B) comparison to finite differences				
Mean	−2.7	−4.4	0.8	−0.8
Standard deviation	6.1	4.3	9.3	7.8
Maximum	13	4	19	13
Minimum	−14	−15	−24	−28
Range enclosing 90% of data	−12 to 8	−12 to 1	−18 to 14	−11 to 9

a Results for the Cleland and Earle, and Hung and Thompson formulae were calculated by the modified forms.
* Ignores 4 experiments in which $T_a > T_{ref}$

For the strawberry example the following data were calculated using Eq. (8) to (13) and Table 3 of Cleland and Earle (1984):

$\Delta H_{18} = 378 \times 10^6$ J/m^3 Bi = 0.673
Ste = 0.196   Pk = 0.114
U = 0.992   P = 0.578
R = 0.156 $(T_{fin} - T_a)/(-18 - T_a) = 0.588$

$t_f = 11.97 \times 1.083$ min $= 12.96$ min.

The freezing time to −18°C is 11.97 min and the time to −25°C is 12.96 min. Results from application of Eq. (7) to (13) to the composite data set in Cleland and Earle (1984) are shown in Tables 1 and 2.

DISCUSSION

THE PREDICTION ACCURACY for the Cleland and Earle formula shown in Tables 1 and 2 is now as good as for any other method for a final center temperature of −18°C, and over the

whole data set, thus vindicating the use of Eq. (1). Correlation with finite difference predictions is also much improved. The improved formula thus meets the criteria in Cleland and Earle (1984). The proposed modification improves the performance of the Hung and Thompson formula at $-10°C$. Correlation with finite difference predictions is now virtually identical at both final center temperatures in the data set. However, because of variations in data from different sources (Cleland and Earle, 1984) agreement with experiments is not even across the whole data set. The similarity in the agreement with finite differences at the two T_{fin} values suggests that the modification may suit the Hung and Thompson formula across a range of final center temperatures.

CONCLUSIONS

A MODIFICATION to the Cleland and Earle freezing time prediction formula is shown to make it as accurate as any other method over the data set studied for final product temperatures that differ from $-10°C$. The prediction accuracy of the Hung and Thompson formula is also improved, by a corresponding modification, for final temperatures other than $-18°C$.

SYMBOLS

- Bi — Biot number hD/k_s
- C_L — volumetric specific heat capacity of unfrozen material ($J/m^{3}°C$)
- C_s — volumetric specific heat capacity of frozen material ($J/m^{3}°C$)
- D — characteristic dimension (full thickness) (m)
- EHTD — equivalent heat transfer dimensionality
- h — surface heat transfer coefficient ($W/m^{2}°C$)
- H — enthalpy (J/m^3)
- ΔH_{10} — enthalpy change in product between T_f and $-10°C$ (J/m^3)
- ΔH_{18} — enthalpy change in product between T_i and $-18°C$ (J/m^3)
- k_s — thermal conductivity of frozen material ($W/m°C$)
- L — latent heat of freezing (derived from ΔH_{10} or ΔH_{18} by subtracting the sensible heat component) (J/m^3)
- r — correlation coefficient
- t — freezing time (sec, min or hr)
- T_a — cooling medium temperature (°C)
- T_f — temperature at which freezing commences (°C)
- T_{fin} — final product center temperature at end of freezing process (°C)
- T_i — initial product temperature (°C)
- T_{ref} — final product temperature used as a reference in empirical freezing formulae (°C)
- U — ratio of temperature driving forces defined in eq. (12)

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4). For the M-Endo-MUG assay, a total confirmatory rate of 90.0% was obtained for the fluorescent colonies (56 out of 62 fluorescent colonies). A false-positive rate of 10.0% was observed (Table 6).

One of the problems commonly encountered during seafood analysis is the presence of stressed or injured microbial cells. Stressed cells have the ability to recuperate and grow on nonselective media, but not on selective media (Clark and Ordal, 1969; Ray and Speck, 1973). The ability of Lauryl Tryptose Broth-MUG (LTB-MUG) to detect heat-stressed cells was examined by Feng and Hartman (1981, 1982). The LTB-MUG system (fluorescence) was superior to the VRB-2 method in detecting heat-injured cells. This is expected, since LTB is a relatively nonselective medium. Perhaps, this is one of the reasons why the LB-MUG assay in this study resulted in higher recoveries of *E. coli* from the seafood samples tested.

The three assays described in this study are rapid (24 hr), sensitive, and save labor and material cost. Any of the three fluoragenic assays can be routinely used by the seafood industry to discriminate seafood samples with high total coliforms and/or *E. coli* (fecal contamination) present.

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International Food Information Service
—Information Retrieval Services

What Is IFIS?

The International Food Information Service (IFIS) was created in 1968 to provide a number of services related to the retrieval of information from the literature of food science and technology.

IFIS's sponsors include the Commonwealth Agricultural Bureaux in England, the Gesellschaft für Information und Dokumentation in the Federal Republic of Germany, the Institute of Food Technologists in the U.S., and the Centrum voor Landbouwpublikaties in Landbouwdocumentatie in the Netherlands.

Primary Services

Food Science and Technology Abstracts (FSTA)

- *FSTA* is a monthly journal containing about 1,500 abstracts. Each issue also contains an Author Index as well as a Subject Index which contains extensive cross-references. All subscribers also receive an annual Author and Subject Index. To produce *FSTA*, IFIS regularly scans 1,400 journals from more than 75 countries, patents from 20 countries, and books in several languages. These abstracts are in English, but are taken from literature originally appearing in as many as 43 languages. A one-year subscription to *FSTA* costs approximately £ 280*.

Magnetic Tapes of *FSTA*

- Everything in *FSTA* is also available on seven- and nine-track IBM magnetic tapes which are for sale at about £ 2,000 per year.
- These tapes are also available for on-line retrieval via Lockheed Information Retrieval Service (USA); SDC Search Service (USA, Canada, France, and Japan); Information Retrieval System (IRS) (Frascati, Italy); or the German Institute for Medical Documentation and Information (DIMDI) (Cologne, Germany).

Secondary Services

Food Annotated Bibliographies

- Food Annotated Bibliographies are selected abstracts from *FSTA* on more than 50 important, specialized aspects of food science and technology. A partial listing of bibliographies available includes: Acidulants in Foods, Colorants, Aflatoxins, Coffee, Stabilizers and Emulsifiers in Foods, Synthetic Dairy Products, and Microwaves in Food Processing.
- Prices range from £ 3 to £ 15 per bibliography.

Current Awareness Service

- Current Awareness or selective dissemination of information services are regular updatings of specifically desired information from each updating of the *FSTA* data base.
- The information is available in three output formats and is carefully selected to match a user's requirements in virtually any aspect of food science and technology.
- Costs are from £ 10 per year for references; £ 15 per year for abstracts.

Retrospective Search Services

- Retrospective searches are searches of the literature to date (the complete data base) for citations and abstracts on specific subjects. More than 100,000 "documents" in *FSTA* are available for search by qualified food science graduates.
- The information is available in three output formats.
- Costs are from £ 5 per volume of *FSTA* searched for references; from £ 7.50 per volume for abstracts. Discounts are available with an annual contract.

Photocopying Service

- Photocopies of any article abstracted in *FSTA* can be quickly made available.
- Cost is 12p per page for single orders, with a minimum charge of £1.50; a deposit account service is also available.
- Translations will be done on request. Prices for this service vary depending upon length of the translation; ask for quote.

*All prices on this page are given in British pounds (£).

**An in-depth analysis of *FSTA* as well as Lockheed and SDC's search services can be found in the article "Retrieving Information from the Food Science Literature," which starts on p. 46 of the September 1977 *Food Technology* (reprints available from IFT at $1 per copy).

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