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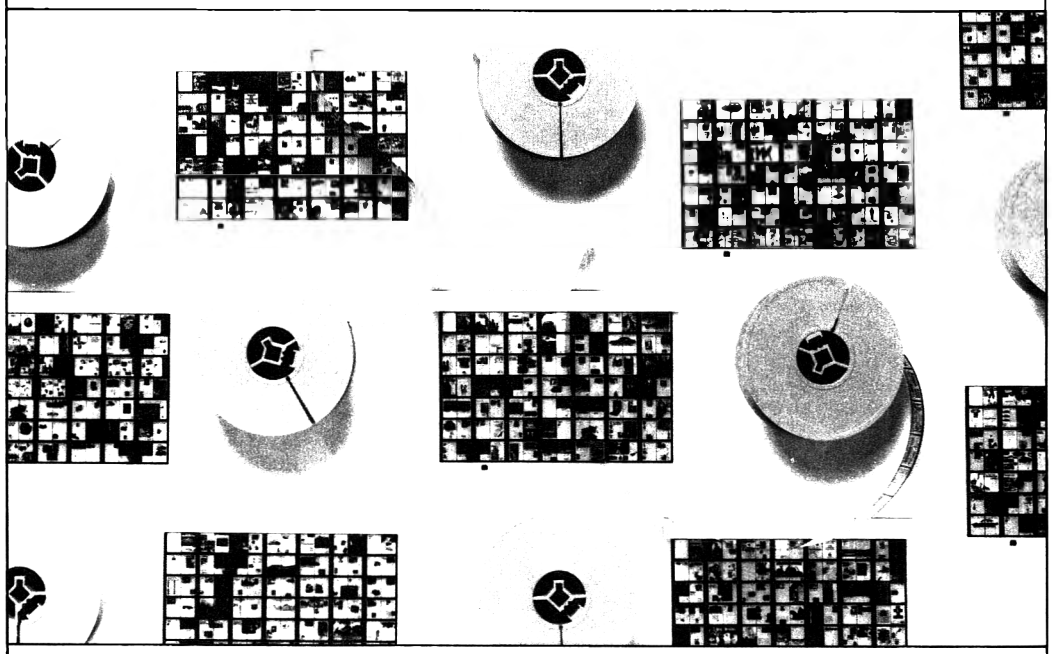

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An Hypothesis Paper

Cheddar Cheese Texture Related to Salt Release During Chewing, Measured by Conductivity—Preliminary Study

FRANCES R. JACK, JOHN R. PIGGOTT, and ALISTAIR PATERSON

ABSTRACT

Salt is dispersed throughout the matrix of Cheddar cheese. We hypothesized that the manner in which salt is released into the mouth during chewing may be directly related to cheese breakdown, and hence texture. Conductivity (used as the best estimate of salt) in the mouth was continually monitored during consumption of a range of Cheddars. Characteristics of conductivity changes varied between cheeses. Relationships were observed between such changes and instrumental and sensory measures of texture. This preliminary study, based on responses of a single subject, suggests the potential of salt release data to help distinguish texture in cheese.

Key Words: chewing, force deformation, sensory/instrumental relations

INTRODUCTION

TEXTURE is of major importance in cheese, being a primary index in quality grading (Bodyfelt, 1988). Although Cheddar cheese must comply with strict compositional and production specifications, consumers readily discriminate between Cheddars in terms of texture (Piggott and Mowat, 1991; Jack *et al.*, 1993b). Hence, the manufacturer must quantify these attributes as accurately as possible. Instrumental measures are available but these often do not provide an adequate representation of texture, as perceived by the consumer. Most texture perception results during chewing. Texture attributes are continually monitored by the consumer as cheese is broken down in the mouth and swallowed. Low correlations between instrumental indices of texture and perception are often due to the effects of chewing being disregarded (Pierson and Le Magnen, 1970). Emphasis is being placed on measuring texture under conditions that closely mimic actual consumption situations. A major area of research has been the study of relationships between chewing patterns and food texture (Jack *et al.*, 1994a). These have varied in complexity from the quantification of chew numbers and frequencies (Harrington and Pearson, 1962; Gacula *et al.*, 1971), to the study of jaw movement (Chew *et al.*, 1988), forces exerted on the teeth during eating (Tornberg *et al.*, 1985), and the measurement of muscle activity (Pierson and Le Magnen, 1970; Eves *et al.*, 1988; Sakamoto *et al.*, 1989).

The production of Cheddar cheese has been extensively described (Banks, 1992). To permit desired microbial and enzymatic activity, salt is not added until after curd formation. Before addition of salt the curd is milled (a fine cutting) and mellowed (rested until curd particle surfaces glisten with exuded whey). The salt is readily dissolved forming a brine (Lawrence and Gilles, 1987). Cheddar cheese has a gel structure, comprising a protein network that entraps fat globules (Prentice, 1987). Brine is dispersed throughout the gel, filling interstices between protein and fat phases. The cheese gel is broken down during chewing, being subjected to repeated fracturing. A hypothesis was suggested; as cheese is consumed breakdown of the matrix results in release of salt into the mouth. Jack *et al.* (1994b) determined that the manner of breakdown varied between Cheddars and that breakdown characteristics were related to changes in perceived texture. We evolved a second hypothesis that since manner of salt release would depend on breakdown characteristics it could be used as a measure of texture. Our objectives were to test these hypotheses, providing a preliminary insight into salt release and its potential as a tool for texture investigation.

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MATERIALS & METHODS

Cheddar cheese samples

Nine Cheddar cheeses were purchased from retail outlets; vintage Canadian Cheddar **VIN**, vegetarian Cheddar **VEG**, reduced fat Cheddar style cheese **RFA**, mild Scottish Cheddar **MDS**, cathedral city Cheddar **CAC**, mild Irish Cheddar **MDI**, vintage English Cheddar **VEN**, half fat Cheddar style cheese **HAF**, mature Scottish Cheddar **MTS**. These represented a wide range of textural characteristics. Cheeses were stored at 4°C. Wrapping in cheese-cloth moistened with a 5% solution of food-grade sodium chloride minimized moisture loss.

Sensory analysis

Descriptive sensory analysis, developed by Stone *et al.* (1974), is widely employed in food research. This approach was used to profile the texture of the cheeses. Samples were rated in terms of nine characteristics: coarse **Co**, creamy **Cre**, crumbly **Cru**, dry **Dr**, grainy **Gr**, hard **Ha**, rubbery **Ru**, smooth **Sm** and sticky **St** (Jack *et al.*, 1994b). The sensory panel consisted of 14 assessors, all staff or students of Strathclyde University. Assessors had

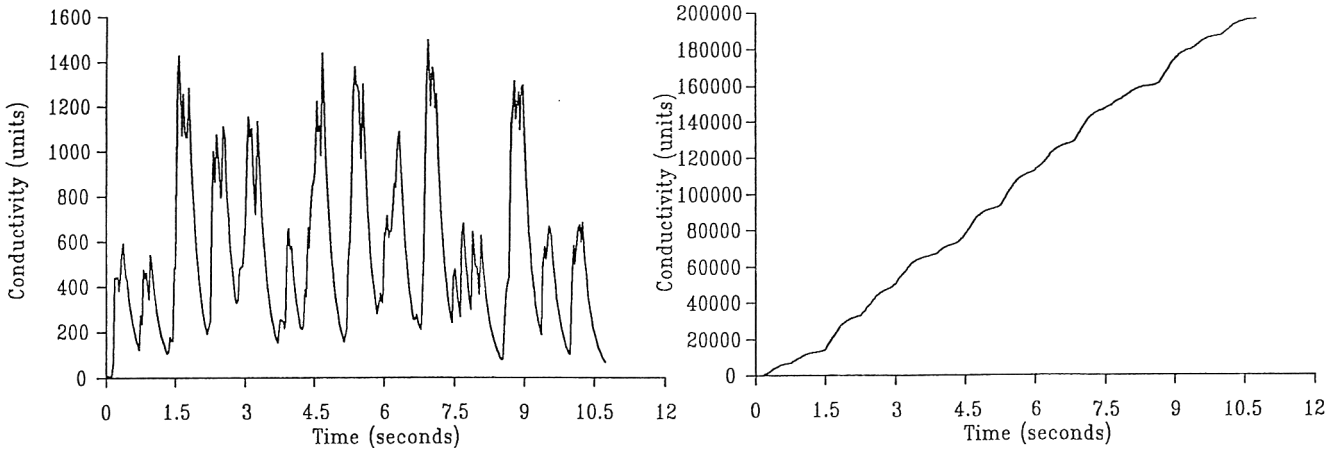

Fig. 1—Typical trace (left) and integrated trace (right) of conductivity in the mouth during consumption of Cheddar cheese.

Table 1—Parameters measured from conductivity traces

Parameter	CODE
Number of peaks	PNUM
Height of first peak	PEAK1
Mean peak height	PMN
Total of peak heights	PTOT
Maximum peak height	PMAX
Number of peaks to max (inclusive)	NMAX
Distance to max (NMAX/PNUM)	DMAX
Duration of chewing (sec)	DUR
Rate of salt release (slope of integrated curve)	RATE

Table 2—Mean force deformation data for nine Cheddar cheeses

Sample	Peak height	Break point	Between curves	A1	A2	A2/A1
VIN	39.7	29.3	69.3	2634	251	0.094
VEG	37.3	51.7	63.7	2861	501	0.176
RFA	35.0	43.0	68.0	2648	411	0.155
MDS	37.3	82.7	57.3	2259	494	0.222
CAC	36.3	38.0	69.7	2778	396	0.143
MDI	27.3	60.3	68.7	1942	315	0.162
VEN	38.6	28.3	73.0	2708	254	0.094
HAF	49.7	73.0	44.3	2698	715	0.267
MTS	24.3	37.3	76.3	2306	293	0.126
psd[a]	4.0	4.1	3.0	283	60	0.02
p<[b]	0.001	0.001	0.001	0.015	0.001	0.001

[a] psd = pooled standard deviation.
[b] p value = probability of rejecting H_0 that there is no difference between samples.

previous training and experience in textural evaluation of Cheddar cheese, being familiar with the analysis of a diverse range of commercial and laboratory produced cheeses. Cylindrical samples of cheese, 15 mm diameter and 20 mm high, were tasted. Samples of these dimensions were also used in measurements of salt release and force-deformation characteristics. All samples were taken at least 3 cm from the outside of the cheese, to minimize effects of surface drying, and cut at 4°C, to prevent barreling. Cheese was allowed to equilibrate at room temperature (≈20°C), for 20 min prior to analysis. Duplicate samples were assessed, with a balanced order of presentation employed. Data were recorded and stored using the PSA-system v1.61 (OP&P Inc., P.O. Box 14167, 3508 SG Utrecht, The Netherlands).

Panel means, calculated for each sensory descriptor, were analyzed by principal components analysis (PCA) (Piggott and Sharman, 1986) using Unscrambler II v3.1 (CAMO A/S, Jarleveien 4, N-7041 Trondheim, Norway). PCA calculates linear combinations of variables (components) describing as much of the variance of the original data as possible. This allows the original multi-dimensional matrix to be simplified without substantial loss of information, and so eases interpretation of complex data matrices. The results of PCA could be graphically displayed as two sets of plots. In the first the correlation of the texture variables with successive components were plotted, to aid interpretation of these components; in the second the sample scores were plotted to show relationships between cheeses.

Instrumental analysis of force-deformation characteristics

Deformation properties were studied using the Instron Universal Testing Machine (Instron Corporation, Canton, MA 02021, USA). Triplicate samples were prepared (Jack *et al.*, 1993a) and subjected to a double, 60% compression at a rate of 20 mm min^{-1}, using a cycling mode. A flat 45 mm diameter probe, lubricated with vegetable oil to minimize frictional effects, was used to effect the compression at a full scale load of 5 kg. The cylindrical samples of cheese were orientated in an up-right column position. The same orientation was used when placing the samples between the teeth in the sensory and salt release experiments. Output from the Instron was in the form of a force-deformation curve. Parameters related to a range of textural characteristics (Jack *et al.*, 1993a) were measured from these curves. These included height of the first peak, distance to break point, distance between curves, areas under first (A1) and second (A2) curves and ratio of areas under curves (A2/A1).

Compositional analysis

Salt (sodium chloride) was determined in triplicate, using the Volhard method and moisture by oven-drying over sand (Kirk and Sawyer, 1991). Amounts of salt in moisture were also calculated.

Salt release

A continuous measure of salt level in the oral cavity was required. Although nonspecific, conductivity was considered to provide the best estimate of salt level, being simple to measure with a relatively nonintrusive sensor. An artificial dental palate with integral electrodes was constructed to allow the monitoring of conductivity (Goff, 1993). These electrodes were comprised of stainless steel foils (5 mm squares by

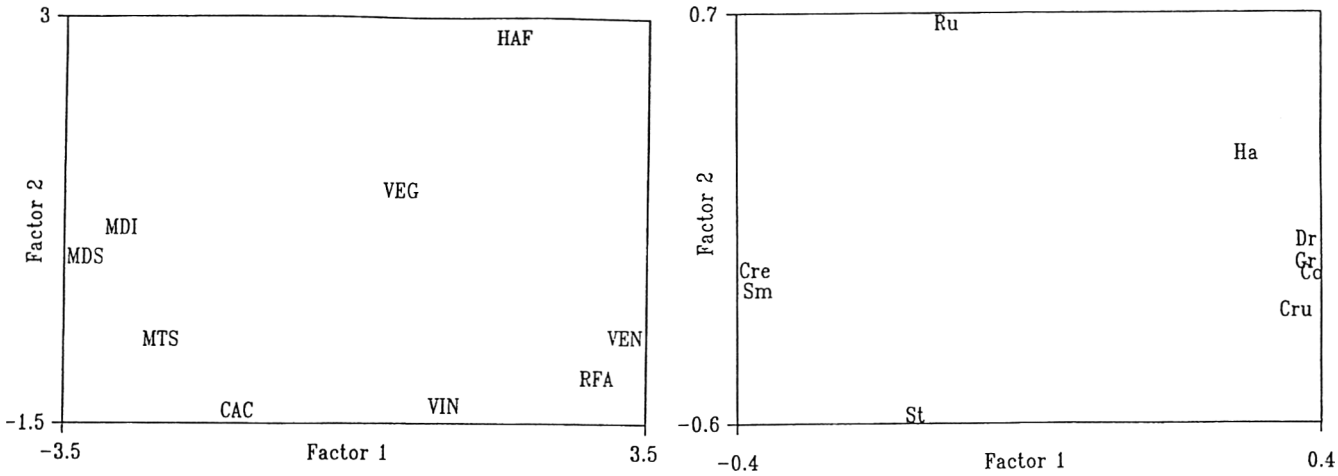

Fig. 2—Sample scores (left) and descriptor loadings (right) on Factors 1 and 2 after PCA of sensory data for nine Cheddar cheeses.

Table 3—Salt and moisture content of Cheddar cheeses

Sample	Salt (g/100g)	Moisture (g/100g)	Salt in moisture
VIN	1.8	32.4	5.6
VEG	1.7	35.6	4.8
RFA	1.5	31.7	4.7
MDS	1.7	36.9	4.6
CAC	1.8	34.0	5.3
MDI	1.6	35.8	4.5
VEN	2.0	30.4	6.6
HAF	2.7	33.9	8.0
MTS	2.3	36.1	6.4
psd	0.1	0.4	0.4
p <	0.001	0.001	0.001

psd = pooled standard deviation.
p value = probability of rejecting H_0 that there is no difference between samples.

Table 4—Salt release data recorded during mastication of nine Cheddar cheeses[a]

Sample	PNUM	PEAK1	PMN	PTOT	PMAX	NMAX	DMAX	DUR	RATE
VIN	15.7	237	1023	15923	1567	6.7	0.46	13.9	17732
VEG	10.7	510	929	9897	1270	6.3	0.59	9.3	17639
RFA	15.7	330	1038	16170	1680	9.0	0.58	14.5	18228
MDS	10.7	397	951	10100	1240	10.0	0.93	9.2	18538
CAC	14.0	483	984	13710	1420	8.0	0.56	11.4	19344
MDI	14.7	610	1081	15893	1423	5.7	0.39	12.5	19871
VEN	14.3	637	1086	15603	1726	7.7	0.52	12.9	19220
HAF	22.3	623	1297	30527	2307	21.0	0.94	18.6	23374
MTS	10.7	583	944	10100	1350	9.7	0.91	8.9	16554
psd[b]	1.4	181	116	2140	183	3.2	0.23	0.9	1581
p <[c]	0.001	0.136	0.031	0.001	0.001	0.001	0.050	0.001	0.004

[a] For codes see Table 1.
[b] psd = pooled standard deviation.
[c] p value = probability of rejecting H_0 that there is no difference between samples.

Table 5—Correlation between force-deformation and salt release parameters[a]

Salt release parameter	Force-deformation: Peak ht	Break point	Between curves	A1	A2	A2/A1
PNUM	0.67	*	*	*	*	*
PMN	0.62	*	−0.62	*	*	*
PTOT	0.68	*	−0.67	*	*	*
PMAX	0.70	*	*	*	*	*
NMAX	0.63	*	−0.80	*	0.78	0.73
DMAX	*	*	*	*	*	0.63
DUR	0.67	*	*	*	*	*
RATE	0.67	*	−0.76	*	0.66	0.65

[a] For codes see Table 1.
* = correlation coefficient < 0.6.

0.25 mm thick) welded to stainless steel polyester insulated conducting wires (0.25 mm diameter). Alginate impressions of the subject's upper and lower dentitions were taken, and articulated on a plain line dental articulator. A simple appliance, similar to an orthodontic plate was constructed, to incorporate the electrodes. This was fabricated of clear acrylic dental base material, with stainless steel retention clasps on the first molars. The appliance fitted closely to the palate facilitating normal chewing. Interference to chewing from the conducting wires was minimal, as they were fine enough to pass between the maxillary lateral incisors and canine teeth.

Conducting wires, from the palate electrodes, were connected to an analogue conductivity meter (CM35, Walden Precision Apparatus Limited, Cambridge, UK). Output was digitized using a 16 channel, A/D, 12-bit converter (PC26AT, Amplicon Liveline, Brighton, UK), installed in a desktop personal computer. The digitized signal was sampled at a frequency of 31 Hz using a data acquisition software package (Microscope, Amplicon Liveline). Conductivity was quantified as arbitrary units. Traces were produced, providing a continuous record of changing conductivity levels in the mouth. A typical conductivity trace, produced during consumption of Cheddar cheese, is shown in Fig. 1. Data were also integrated, via continuous summation of the data points (Fig. 1). Several parameters were measured from these conductivity traces (Table 1).

The salt release experiment employed a single subject, of sound dentition. Cheeses were prepared as before. Conductivity in the oral cavity was continuously recorded from the onset of chewing through swallowing. Rest periods, of 5 min between samples, minimized effects of fatigue. Samples were examined in triplicate. A balanced order

Table 6—Correlation between compositional and salt release parameters[a]

Salt release parameter	Salt	Moisture	Salt in moisture
PNUM	*	*	0.62
PMN	0.60	*	0.69
PTOT	0.61	*	0.69
PMAX	0.66	*	0.78
NMAX	0.81	*	0.76
DMAX	0.63	*	*
DUR	*	*	*
RATE	*	*	*

[a] For codes see Table 1.
* = correlation coefficient < 0.6.

of sample presentation was employed. The subject was positioned away from the computer monitor, and could not see the conductivity traces as they were generated.

A baseline recording was made by having the subject repeat the chewing patterns used when consuming Cheddar cheese, in this case with the mouth empty. Small fluctuations in conductivity were observed, due to movement of saliva around the mouth. Effects of saliva were negligible, in comparison with levels of conductivity resulting on consumption of cheese, and therefore no baseline was deducted.

Statistical analysis

Correlation coefficients between conductivity parameters, force-deformation, compositional and sensory data were calculated. The potential of salt release data as a predictor of sensory texture perception was explored using partial least squares regression (PLS) (Unscrambler II v3.1). In this technique a few orthogonal latent variables (factors) were sought to describe inter-relationships between two blocks of variables, with primarily those factors relevant to prediction of the regressed matrix (Y) being extracted from the regressor matrix (X). Thus PLS provided pairs of directions, one from each set, which showed strong covariance with each other. Cross-validation was used to minimize over-fitting of data (Martens and Martens, 1986).

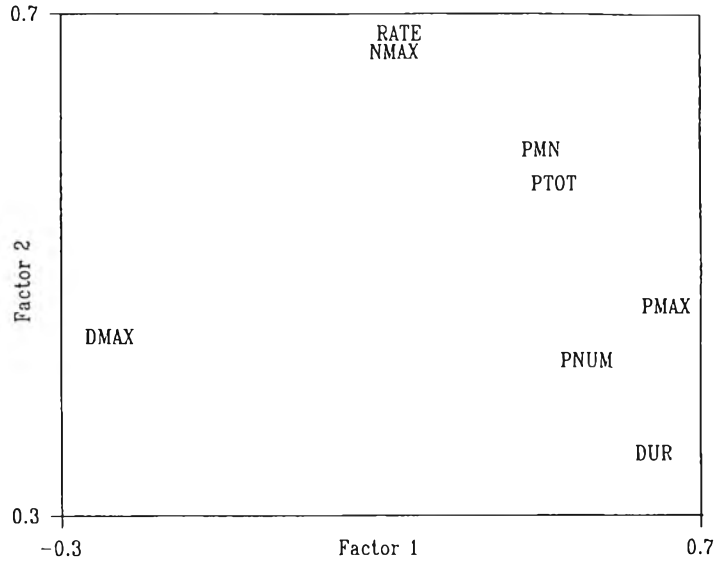

Fig. 3—Plot of correlation coefficients between salt release parameters and Sensory Factors 1 and 2. Abbreviations are shown in Table 1.

RESULTS & DISCUSSION

THE SENSORY PANEL successfully discriminated between the Cheddars in terms of texture. Two factors, shown by analysis of variance on replicates to discriminate between cheeses ($p<0.001$), were produced from the PCA of sensory data (Fig. 2). These accounted for 65% and 21% of variance. Primarily dry, coarse, grainy Cheddars were separated from smooth, creamy ones (Factor 1). Rubberiness was also important, Factor 2 separating rubbery from sticky samples. The cheeses also displayed significantly different force-deformation characteristics (Table 2) and compositions (in terms of salt, moisture and salt in moisture) (Table 3).

Typical conductivity traces produced during consumption of Cheddar cheese (Fig. 1) were composed of a series of peaks. Each peak related to a single chew stroke. This suggested that the initial hypothesis was correct; that salt was released into the mouth as the cheese matrix was broken down during chewing. The assumption that this effect was due to salt from the cheese, rather than salt from saliva, was supported by the fact that the baseline (produced with no sample) resulted in a negligible trace. The integrated trace (Fig. 1) displayed a uniform increase, illustrating that salt was released at a steady rate during chewing.

Characteristics of the conductivity trace differed among cheeses. Each of the measured parameters, except for the height of the first peak (**PEAK1**), showed significant differences (Table 4). This non-discriminatory parameter was removed prior to further analysis. Correlations between conductivity in the mouth and the force-deformation and compositional characteristics of the cheese were observed (Tables 5 and 6). Two force-deformation characteristics, *distance to break point* and *area under first curve*, were not related to any conductivity parameters. The number of significant correlations between conductivity parameters and remaining force-deformation characteristics varied between 1 and 4. Peak height of force deformation curves significantly correlated with all of conductivity parameters, except **DMAX** (Table 5). Since peak height is a measure of hardness (Lee *et al.*, 1978), results suggested that hardness and manner of salt release were related. The salt release parameters did not correlate with levels of moisture in the cheese, though significant correlation with both total salt, and salt in moisture were observed (Table 6). As expected the height of the conductivity peaks generally increased with increasing levels of salt in the cheese. This also supported the hypothesis that changes in conductivity in the mouth during chewing were a result of salt being released from the cheese gel.

Relationships were observed between conductivity in the mouth and perceptions of texture. Correlation coefficients between conductivity parameters and Sensory Factors 1 and 2 were plotted (Fig. 3). **PNUM, PMN, PTOT, PMAX** and **DUR** were located on the positive end of Factor 1, correlated with hard, dry textures. Three of these parameters (**PNUM, PTOT** and **DUR**) were influenced by the number of chews in the consumption sequence. Hence cheeses with hard, dry characteristics (e.g., vintage English Cheddar and reduced fat Cheddar style cheese) required the most mastication. The parameters **PMN, PMAX** and **PTOT** all related to height of peaks produced. The higher the values of these parameters the more salt was being released per chew. Hence most salt was released from hard, dry cheeses on each chew stroke. This may have been related to the lower moisture, and subsequent higher salt in moisture ratios, in dry cheeses. **DMAX** was related to the negative end of Sensory Factor 1, the duration of chewing required to reach maximal salt release being directly related to sample smoothness and creaminess.

The remaining two conductivity parameters (**NMAX** and **RATE**) were most closely related to the positive end of Sensory Factor 2, rubberiness. Both of these parameters related to the rate at

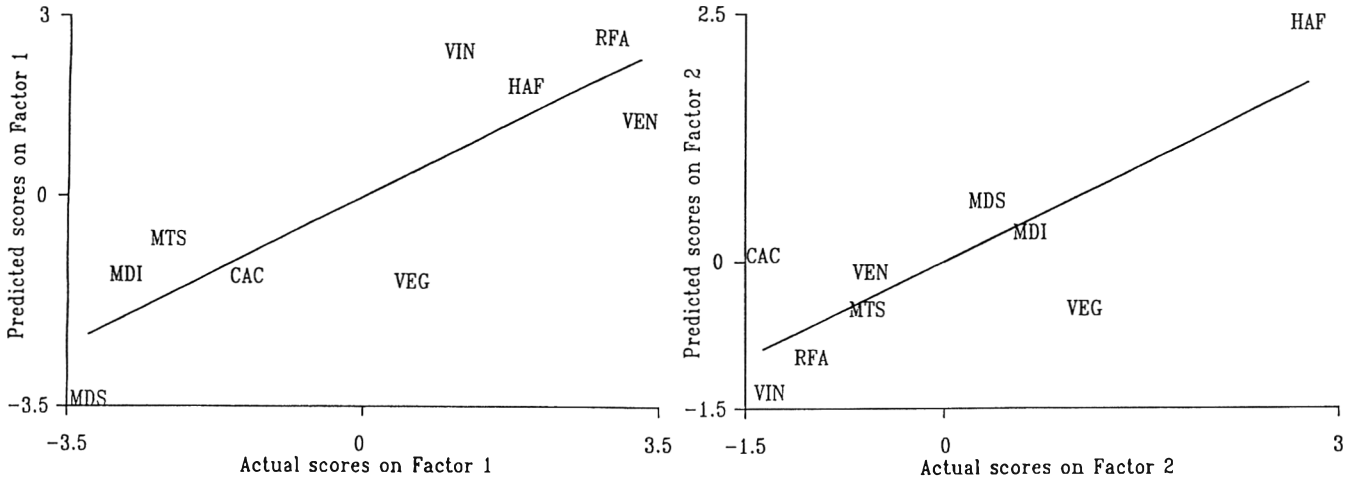

Fig. 4—PLS predictions of Sensory Factors 1 and 2 using salt release data.

which salt was released from the cheese as it was chewed. Rubbery cheeses (such as the half fat Cheddar style cheese) displayed slow rates of salt release. Such results suggest that disintegration of the gel on each chew stroke was less extensive in these cheeses than in other Cheddars.

Using PLS to model relationships, salt release was a good predictor of Sensory Factors 1 and 2 (Fig. 4). Correlation coefficients of 0.843 and 0.812, respectively, were observed. Two factors were optimum for prediction in each case. None of the cheeses was an obvious outlier. However, the sensory characteristics of the vegetarian Cheddar were not as well modeled as the other cheeses, although prediction errors were similar. Differences in protein hydrolysis patterns, due to use of a microbial coagulant in that product, may thus have significantly affected gel structure, and hence salt release character. This should be explored further.

CONCLUSIONS

CONDUCTIVITY IN THE MOUTH during consumption of Cheddar cheeses can be used as an estimate of salt release from the cheese matrix. Salt release characteristics can be used as predictors or indicators of textural quality. This preliminary study has limitations. Further development and validation are required, particularly to examine replication by other subjects. The observation that salt release is significantly influenced by gel structure (texture) suggests that there may be similar relationships between texture and flavor release in cheese.

REFERENCES

Banks, J.M. 1992. Cheese. In *The Technology of Dairy Products*. R. Early (Ed.), p. 39–65. Blackie and Sons Ltd., Glasgow, UK.

Bodyfelt, F.W. 1988. Sensory evaluation of cheese. In *The Sensory Evaluation of Dairy Products*. F.W. Bodyfelt, J. Tobias, and G.M. Trout (Ed.), p. 300–375. Van Nostrand Reinhold, New York.

Chew, C.L., Lucas, P.W., Tay, D.K.L., Keng, S.B., and Ow, R.K.K. 1988. The effect of food texture on the replication of jaw movement in mastication. J. Dentistry 16: 210–214.

Eves, A., Boyar, M., and Kilcast, D. 1988. Electromyographic evaluation of the texture of confectionery products. In *Food Acceptability*, D.M.H. Thomson (Ed.), p. 459–472. Elsevier, London.

Gacula, M.C., Reaume, K.J., and Luckett, R.L. 1971. Statistical aspects of the correlation between objective and subjective measurements of meat tenderness. J. Food Sci. 36: 185–189.

Goff, T. 1993. Personal communication.

Harrington, G. and Pearson, A.M. 1962. Chew count as a measure of tenderness of pork loins with various degrees of marbling. J. Food Sci. 27: 106–110.

Jack, F.R., Paterson, A., and Piggott, J.R. 1994a. Review: Understanding and quantifying texture perception in relation to product development. Int. J. Food Sci. Technol. (Submitted).

Jack, F.R., Paterson, A., and Piggott, J.R. 1993a. Relationships between rheology and composition of Cheddar cheeses and texture as perceived by consumers. Int. J. Food Sci. Technol. 28: 293–302.

Jack, F.R., Piggott, J.R., and Paterson, A. 1994b. Analysis of textural changes in hard cheese during mastication by progressive profiling—a novel sensory strategy. J. Food Sci. 59: 539–543.

Jack, F.R., Piggott, J.R., and Paterson, A. 1993b. Discrimination of texture and appearance in Cheddar cheese using consumer Free-Choice Profiling. J. Sens. Stud. 8: 167–176.

Kirk, R.S. and Sawyer, R. 1991. *Pearson's Composition and Analysis of Foods*. 9th ed. Longman Scientific and Technical, London.

Lawrence, R.C. and Gilles, J. 1987. Cheddar cheese and related dry-salted varieties. In *Cheese: Chemistry, Physics and Microbiology, Vol. 2: Major Cheese Groups*. P.F. Fox (Ed.), p. 1–44. Elsevier, London.

Lee, C.H., Imoto, E.M., and Rha, C. 1978. Evaluation of cheese texture. J. Food Sci. 43: 1600–1605.

Martens, M. and Martens, H. 1986. Partial Least Squares Regression. In *Statistical Procedures in Food Research*, J.R. Piggott (Ed.), p. 293–359. Elsevier, London.

Pierson, A. and Le Magnen, J. 1970. Study of food texture by recording chewing and swallowing movements. J. Text. Stud. 1: 327–337.

Piggott, J.R. and Mowat, R.G. 1991. Sensory aspects of maturation of Cheddar cheese by descriptive analysis. J. Sens. Stud. 6: 49–62.

Piggott, J.R. and Sharman, K., 1986. Methods to aid interpretation of multidimensional data. In *Statistical Procedures in Food Research*, J.R. Piggott (Ed.), p. 181–232. Elsevier, London.

Prentice, J.H. 1987. Cheese rheology. In *Cheese Chemistry, Physics and Microbiology*, Vol. 1, P.F. Fox (Ed.), p. 299–344. Elsevier, London.

Sakamoto, H., Harada, T., Matsukubo, T., Takaesu, Y., and Tazaki, M. 1989. Electromyographic measurement of textural changes of foodstuffs during chewing. Agric. Bio. Chem. 53: 2421–2433.

Stone, H., Sidel, J., Oliver, S., Woolsey, A., and Singleton, R.C. 1974. Sensory evaluation by Quantitative Descriptive Analysis. Food Technol. 28: 24–34.

Tornberg, E., Fjelkner-Modig, S., Ruderus, H., Glantz, P., Randow, K., and Stafford, D. 1985. Clinically recorded masticatory patterns as related to the sensory evaluation of meat and meat products. J. Food Sci. 50: 1059–1066.

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Aroma Extract Dilution Analysis of Aged Cheddar Cheese

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ABSTRACT

Volatile compounds were isolated from 3-yr-old Cheddar cheese by molecular distillation. They were analyzed by aroma extract dilution analysis, a combination of gas chromatography and olfactometry that reveals the aroma volatiles with the highest odor potency (the highest ratio of concentration to odor threshold). The identified compounds with highest odor potency were listed in order of elution on a DB-wax capillary column. Their potencies and retention indices (in parentheses) were: ethyl acetate (3, 882), 2-methylbutanal and 3-methylbutanal (81, 899), diacetyl (3, 960), α-pinene (3, 989), ethyl butyrate (243, 1018), ethyl caproate (81, 1218), 1-octen-3-one (9, 1284), acetic acid (27, 1418), methional (9, 1428), propionic acid (81, 1517), butyric acid (729, 1616), valeric acid (27, 1736), caproic acid (81, 1845), capric acid (9, 2198) and lauric acid (9, 2580).

Key Words: Cheddar Cheese, aroma, extract dilution, acceptability

INTRODUCTION

AROMA EXTRACT DILUTION ANALYSIS (AEDA) combines gas chromatography and olfactometry to determine which volatile compounds are most potent in food. Two equivalent procedures for AEDA have been described. "Charm" (Acree et al., 1984), and FD (Flavor Dilution) (Ullrich and Grosch, 1987). In both, flavor extracts are injected into a gas chromatograph and subjects smell the resulting peaks as they elute. They smell the extracts at different dilutions to provide references for the relative potency of individual compounds. Those that require much dilution before becoming undetectable are most potent. They have high CHARM values (Acree et al., 1984) or FD factors (Ullrich and Grosch, 1987).

CHARM values and FD factors are similar to the "odor units" described by Guadagni et al. (1966). They are estimates of relative odor potency. The "odor unit" is defined as the ratio of the concentration of a compound in a food to its odor threshold in that food. It differs from AEDA units because they are determined for aroma extracts, not in the food itself. Interactions with the food matrix do not exert much influence on them.

The aroma of Cheddar cheese has been investigated many times. Maarse and Visscher (1989) listed 213 compounds that had been isolated and identified in 50 different reports. However, only a fraction of those compounds contributes to the aroma of aged Cheddar cheese. Our objective was to use AEDA to identify the most potent aroma volatiles.

MATERIALS & METHODS

Materials

Aged Cheddar cheese was purchased on the retail market. It was selected because it had a flavor that was very strong, yet typical of aged Cheddar cheese and free of defects.

Extract preparation

The cheese was trimmed and 2,700g were cut by hand into ≈2 cm cubes and centrifuged to remove the oil. Centrifugation was at 40°C and 2,500 × *g* for 2 hr (International Equipment Company, model PR-J, Needham, Ma).

The recovered oil (700g) was subjected to molecular distillation using a Pope Scientific, Inc. (Menomonie Falls, WI) ≈5 cm wiped film still equipped with two cold traps. The cold finger was cooled with ice water, the cold traps were filled with liquid N_2; the vacuum was set at 680 mmHg and the jacket was heated to 50°C. Oil was degassed and then distilled at an average rate of ≈2 mL/min. When distillation was complete, the cold finger was rinsed with 25 mL diethyl ether and 50 mL water. The cold trap was allowed to warm to ≈0°C before it was also rinsed with 25 mL of ether and 50 mL of water. All fractions were combined in a 250 mL separatory funnel and allowed to separate. The aqueous fraction was decanted. Then the organic fraction was dried with $MgSO_4$ and evaporated to ≈0.2 g under a stream of dry N_2.

AEDA

A Hewlett-Packard (Avondale, PA) model 5880A gas chromatograph (GC) was equipped with a split/splitless injection port, a 30 m × 0.25 mm i.d. × 0.25 μm film thickness DB-Wax capillary column (J. & W. Scientific, Rancho Cordoba, CA) and a Chromfit (Chromtech, Inc., Apple Valley, MN) "Y" connector, allowing the effluent to be split between a sniffing port and a flame ionization detector (FID). A 1 μL injection of extract was made in the splitless mode with injection port 250°C and initial oven 20°C held for 1 min. The temperature was then increased at 5°C/min. to 230°C.

Dilutions for AEDA analysis were done sequentially by volume (1:3). To help minimize bias, extracts were sniffed starting with the most dilute sample (FD number of 3^{10}, or 59,049) working toward the less diluted and finally the undiluted extract. Sniffing was done by two experienced subjects working independently. The beginning and end times

Table 1—Compounds sniffed in the GC effluent

No.	RI[a]	Identity	Notes[b]
1	880–884	Ethyl acetate	GC, MS,SM
2	894–903	2-methylbutanal or 3-methylbutanal	GC, SM
3	957–962	Diacetyl	GC, SM
4	989	a-pinene	GC, SM
5	1010–1025	Ethyl butyrate	GC, MS,SM
6	1080		
7	1100–1106		
8	1182–1197		
9	1214–1221	Ethyl caproate	GC,MS,SM
10	1281–1287	1-octen-3-one and acetoin	GC, SM
11	1341–1350		
12	1355		
13	1361–1367		
14	1412–1425	Acetic acid	GC, MS,SM
15	1427–1429	Methional	GC, SM[c]
16	1466–1507	Acetic acid (ending)	GC, SM
17	1514–1520	Propionic acid and 2,3-butanediol	GC, MS[c]
18	1521–1547		
19	1598–1633	Butyric acid	GC, MS,SM
20	1713–1759	Valeric acid	GC, MS[c]
21	1803–1811		
22	1830–1860	Caproic acid	GC, MS, SM
23	1861–1881		
24	2194–2302	Capric acid	GC, MS[c]
25	2574–2585	Lauric acid	GC, MS, SM

[a] RI calculated from sniffing and FID results on GC. A range is given when odors persisted for more than one RI unit. [b] GC-authentic standard eluted within range of retention times consistent with unknown odor. MS-high quality match obtained between mass spectrum of unknown peak and appropriate reference. SM-named compound had same odor character as authentic standard. [c] Peaks had variable descriptors and were quite broad.

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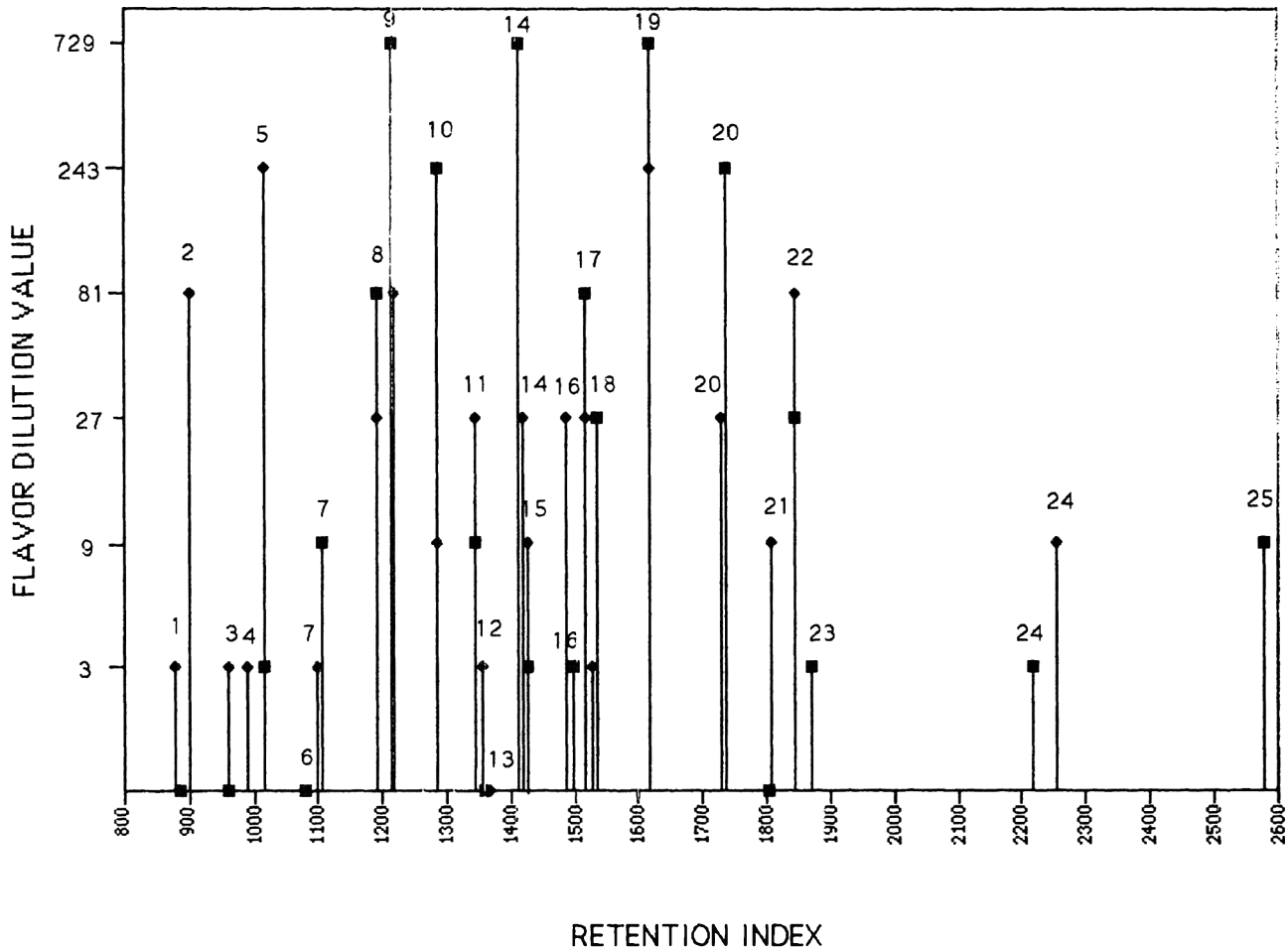

Fig. 1—Combined aromagram results for two judges. The height of the lines corresponds to highest flavor dilution reached by Judge 1 (■) and Judge 2 (◆). Peak numbers, see Tables 1 and 2. In peaks 6 and 13 results for the two judges were identical, so only results of Judge 1 were clearly visable.

of aromas were recorded on strip charts that were synchronized with the FID chromatograms.

Identification of aroma volatiles

A Hewlett-Packard model 5890 GC coupled to a Hewlett-Packard 5970 mass spectrometer (GC-MS) was used to determine spectra of the odoriferous peaks. A similar column was used as in the AEDA and under identical conditions. Tentative identifications were provided by match of experimental mass spectra with reference mass spectra.

A Hewlett-Packard model 5921 A atomic emission detector (AED) was used in conjunction with a model 5890 Series II GC. The same DB-Wax capillary column was used as in the sniffing and FID experiments under the same conditions. The AED was adjusted to be sensitive to C, N and S.

Straight-chain hydrocarbons were determined on both systems to simplify peak matching. Retention indices (RI) were computed for compounds and published lists of RI (Jennings and Shibamoto, 1980; Sadtler, 1986) were consulted for confirmation.

RESULTS & DISCUSSION

ALL BUT ONE OF THE PEAKS (Table 1) have been reported before in Cheddar cheese. The exception was the "mushroom" peak, tentatively identified as 1-octene-3-one. This compound was previously associated with metallic flavor in milk (Swaboda and Peers, 1977). It had a sensory threshold of 0.8 to 2.2 ppb (Fischer and Grosch, 1987), which helps explain why no trace of this compound was evident on the GC-MS. It is one of two compounds listed by Le Loch-Bonazzi and Wolff (1991) isolated from mushrooms with a specific "mushroom" aroma. The other such compound was 1-octene-3-ol. Fischer and Grosch (1987) reported (in two separate determinations) RI values of 1291 and 1294 for 1-octen-3-one, and 1447 and 1448 for 1-octen-3-ol. Our RI values, under different conditions, were: the mushroom aroma, 1283; authentic 1-octen-3-one, 1285; authentic 1-octen-3-ol, 1420. Both sniffers confirmed that authentic 1-

Table 2—Judges' descriptors of peaks which could be reliably[a] sniffed in the GC effluent (Listed by Peak No., Table 1)

No.	Judge 1	Judge 2
1	Juicy Fruit gum	Apples
2	Tootsie Rolls	
3	Butter	Butter, mild Cheddar
4	Pine, green	
5	Sweet, fruity	Apple blossom
6	Plastic	Glue
7	Cooked milk	Oxidized, glue
8	Sulfur	Potato, cardboard
9	Fruit melon	Sour fruit
10	Mushroom, smoky	Mushroom, metallic
11	Burnt sugar or garlic	Garlic, "sickly sweet"
12	Oxidized, glue	
13	Green, floral	Geraniums
14	Peppers, green	Green, fruity, floral
15	Cooked milk, acrid	Sulfur, oil, onions
16	Green	Pungent, green, cereal
17	Gas, burnt, sulfur	Sulfur, cloves, solvent
18	Green, wood, vegetable	Green, cardboard, glue
19	Nacho Cheese	Cheese, toasted cheese
20	Rain. wood, vegetable	Spicy, nutty, grain
21	Acrid	Toasted, cardboard
22	Bad breath, popcorn	Goaty, free fatty acid
23	Peppers, green and red	
24	Warm, stale butter	Sour fruit
25	Soapy	

[a] Criterion for reliability was that the peak should be detected in two separate trials.

octen-3-one and the unknown at 1283 had the same odor character. Therefore, we consider the evidence strong that 1-octen-3-one was identified, though no mass spectrum could be obtained.

In some cases aromas at certain times appeared to derive from more than one compound. Methional, for example, was tentatively identified in the middle of the acetic acid peak, because a potato-like smell was reported there and authentic methional, (which has a potato-like smell) eluted at that time.

The descriptors the two judges chose for propionic acid strongly indicated the presence of a sulfur compound, but no trace of sulfur could be found, even using the AED. Producing descriptors is highly subjective (Table 2). Possibly the 2 judges simply found propionic acid difficult to describe out of context. Identifications were reinforced by published RI data, authentic standards and, in some cases, mass spectral matches. However they must be considered tentative until repeated on a second GC column of differing polarity. This should reveal whether smells truly resulted from the identified peaks, or whether their appearances at the same retention times were due to coincidence.

Aromagrams were developed (Fig. 1) which combined sniffing results of the two judges. To our knowledge, this is the first instance in which AEDA has been applied to Cheddar cheese. The judges appeared to differ in sensitivities to certain compounds. From the aromagram, the aroma of Cheddar cheese does not appear simple.

A definitive list of potent odorants in Cheddar cheese, if constructed, would probably contain more than 25 compounds. The method of sample preparation did not allow recovery of the very low-boiling components of the cheese aroma, for example, hydrogen sulfide, acetaldehyde or methanethiol (Libbey et al., 1963). Also possibly some intense peaks of long duration were due to more than one compound.

Frijters (1978) noted that odor units were not necessarily proportional to aroma intensity. Conceivably compounds that could not be perceived after the first dilution were more intense in the cheese than other compounds that were detectable after many dilutions. In spite of this theoretical premise, we could conclude that compounds that could be smelled were more likely to be important to the aroma of Cheddar cheese than those which could not. In addition, many compounds that might usually be in this "medium volatility" fraction of Cheddar cheese aroma can now be considered of little importance.

CONCLUSIONS

AROMA EXTRACT DILUTION ANALYSIS was helpful to narrow the group of compounds that should be considered important to the chemical nature of Cheddar cheese aroma. The methodology did not allow evaluation of the most volatile fractions of Cheddar cheese aroma. The relative importance of hydrogen sulfide, methanethiol or acetaldehyde among the potent odorants remains to be determined.

REFERENCES

Acree, T.E., Barnard, J., and Cunningham, D.G. 1984. A procedure for the sensory analysis of gas chromatographic effluents. Food Chem. 14: 273–286.
Fischer, K.H. and Grosch, W. 1987. Volatile compounds of importance in the aroma of mushrooms (*Psalliota bispora*) Lebensmittel, Wissenschaft und Technologie 20: 233–236.
Frijters, J.E.R. 1978. A critical analysis of the odour unit number and its use. Chemical Senses & Flavour 3: 227–232.
Guadagni, D.G., Buttery, R.G., and Harris, J. 1966. Odour intensities of hop oil components. J. Sci. Fd. Agric. 17: 142–144.
Jennings, W. and Shibamoto, T. 1980. *Qualitative Analysis of Flavor and Fragrance Volatiles by Glass Capillary Gas Chromatography*. Academic Press, New York.
Le Loch-Bonazzi, C. and Wolff, E. 1991. Characterization of the flavour properties of the cultivated mushroom (*Agaricus bisporus*) and the influence of drying processes. Lebensm. Wiss. Technol. 24: 386–390.
Libbey, L.M., Bills, D.D., and Day, E.A. 1963. A technique for the study of lipid-soluble food flavor volatiles. J. Food Sci. 28: 329–333.
Maarse, H. and Visscher, C.A. (Eds.). 1989. *Volatile Compounds in Food*, 6th ed., Vol. I, p. 339–345. TNO-CIVO Food Analysis Institute, Zeist, Holland.
Sadtler Research Laboratories. 1986. *The Sadtler Standard Gas Chromatography Retention Index Library*, Vol. 4, p. 591–637. Sadtler Research Laboratories, Philadelphia.
Swaboda, P.A.T. and Peers, K.E. 1977. Metallic odour caused by vinyl ketones formed in the oxidation of butterfat. The identification of octa-1,cis-5-dien-3-one. J. Sci. Fd. Agric. 28: 1019–1024.
Ullrich, F. and Grosch, W. 1987. Identification of the most intense odor compounds formed during autoxidation of linoleic acid. Z. Lebensmit. Unters. Forsch. 184: 277–282.
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Chemical Pretreatment and Microfiltration for Making Delipidized Whey Protein Concentrate

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ABSTRACT

Chemical pretreatment, microfiltration (MF) and ultrafiltration (UF) were applied to produce delipidized whey protein concentrates (WPC). Processes including both chemical pretreatment and MF resulted in WPC with <0.5% lipids. Low-pH UF and isoelectric point (pI) precipitation were more effective for lipid removal than chemical pretreatment by thermocalcic aggregation. Protein permeation ratios in MF processes were improved by UF preconcentration of whey. Protein permeation and flux were different between the two MF membranes used. Isoelectric point precipitation increased β-Lg contents, but not α-La, in the resulting WPC (B). Minor proteins exhibited lower concentrations in WPC B and MF WPC products.

Key Words: dairy, whey protein, delipidized concentrate, thermocalcic aggregation, microfiltration

INTRODUCTION

ANNUAL U.S. PRODUCTION of spray-dried whey protein concentrates (WPC) that contain ≥35% protein is in excess of 200 million pounds (American Dairy Products Institute, 1993). WPC are used as nutritional and functional ingredients in food products. Residual whey lipids (RWL), i.e., small milkfat globules, and phospholipoprotein complex (PLPC), impair ultrafiltration (UF) membrane flux during WPC manufacture (Lee and Merson, 1976; Hiddink et al., 1981; Kuo and Cheryan, 1983; Patocka and Jelen, 1987), thus resulting in WPC with 4 to 6% total lipids (Morr and Foegeding, 1990). The RWL impair functionality (De Wit et al., 1986; Maubois et al., 1987; Kim et al., 1989; Morr and Foegeding, 1990; Morr, 1992), and promote off-flavor development during storage (Morr and Ha, 1991; Laye, 1993; Laye et al., 1994) of WPC products.

Since commercial milk separators do not completely remove RWL from cheese whey, additional processing is required to manufacture delipidized WPC with ≤1% RWL (Morr and Ha, 1993). Several chemical pretreatment and membrane processing methods for further delipidization of cheese whey have been proposed. Breslau et al. (1975) suggested a whey delipidization process based on UF at pH 3 and isoelectric precipitation of lipoproteins. A thermocalcic delipidization pretreatment developed by Attebery (1971) involved addition of divalent metal ions, pH adjustment and heating to enhance aggregation and removal of PLPC. It was evaluated by Fauquant et al. (1985), Pierre and Fauquant (1986), and Maubois et al. (1987) and modified for continuous operation by Lehmann and Wasen (1990). The resulting PLPC aggregate has been removed from the whey by cross-flow microfiltration (MF) (Maubois et al., 1987), by centrifugal clarification (Kim et al., 1989), and by perpendicular flow MF (Rinn et al., 1990).

Although MF effectively removes RWL from cheese whey (Merin et al., 1983), it has generally led to incomplete recovery of proteins in the delipidized permeate fraction (Hanemaaijer, 1985; Knopp, 1992). Those studies indicated that chemical pretreatment was required to obtain satisfactory membrane flux and protein permeation.

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Our objective was to investigate the use of microfiltration and chemical pretreatments to manufacture delipidized WPC with minimal RWL, and to explore the effectiveness of chemical pretreatment to improve MF membrane flux and protein permeation.

MATERIALS & METHODS

Whey

Commercial Swiss cheese whey was obtained from Holmes Cheese Co. (Millersburg, OH). The milk was HTST-pasteurized at 72°C prior to setting. The whey was drained from the cheese vat, processed through a commercial Westfalia milk clarifier/separator and cooled to 0–5°C. The whey, which contained 0.43 g/L calcium and 0.42 g/L phosphorus, and had an initial pH of 6.5–6.6, was adjusted to pH 6.0 with concentrated HCl and HTST-pasteurized at 72°C, cooled and stored at 0–5°C.

Whey processing

Two MF membranes were used: (1) a Romicon PM500 (500,000 MW cut-off, pore size 0.1 μm) polysulfone tubular hollow-fiber (HF) membrane with 1.3 m^2 surface area (Romicon Inc., Woburn, MA); and (2) a 0.135 m^2 surface area ceraflo α-alumina ceramic membrane with pore size 0.45 μm (APV Crepaco, Wilmington, MA). Inlet and outlet module pressures were 110.3 kPa and 68.9 kPa, respectively, for both membranes. Ultrafiltration (UF) was performed with a Romicon PM10 (10,000 MW cut-off), polysulfone tubular HF membrane with 2.3 m^2 surface area. Inlet and outlet module pressures were 110.3 kPa and 82.7 kPa, respectively.

MF and UF membrane flux were determined at the beginning of each process and after each 10 L of permeate had been collected and expressed as L $m^{-2}h.r^{-1}$. Final UF retentates were spray-dried with a Lab S-1 spray dryer (Anhydro Inc., Attleboro Falls, MA) equipped with a rotary atomizer nozzle operated at 33,000–37,000 rpm. Inlet air temperature was 190°C and outlet 80–84°C.

Chemical pretreatment of whey

HTST-pasteurized, cooled and stored cheese whey was chemically pretreated in ≈38 L stainless steel milk cans by a method similar to that of Attebery (1971), as modified by Fauquant et al. (1985). Formation of insoluble calcium phosphate aggregates in the whey was achieved by adjusting the Ca concentration to 1.44g L^{-1} with 0.1M $CaCl_2$, warming to 50°C, and adjusting to pH 7.3 with 6 N NaOH. The pretreated whey was usually held overnight to allow calcium phosphate aggregates with associated RWL to settle and produce a clear supernatant layer.

WPC manufacture

Six different WPC, i.e., A, B, C, P, MF 0.1, and MF 0.45 were produced (See Figs. 1, 2, and 3). Products were made in duplicate from whey obtained on different days over a period of 4 to 5 wk. All membrane processing was done at 45 ± 5°C. The processes were designed to produce about 5L of final retentate. Diafiltration (DF) steps were performed by adding deionized water to maintain constant volume.

WPC C was made from pH 6 pasteurized whey by conventional UF and DF processing (Fig. 1). WPC A was made from pasteurized whey adjusted to pH 3.0 with concentrated HCl by conventional UF and DF processing (Fig. 1). WPC B was made by the method of Breslau et al. (1975) which involved UF at pH 3 and pH 4.6, isoelectric precipitation of PLPC (Fig. 2). WPC P was made from whey pretreated by the thermocalcic method of Fauquant et al. (1985) to remove PLPC (Fig. 2). Both WPC MF 0.45 and MF 0.1 were made from whey pretreated by

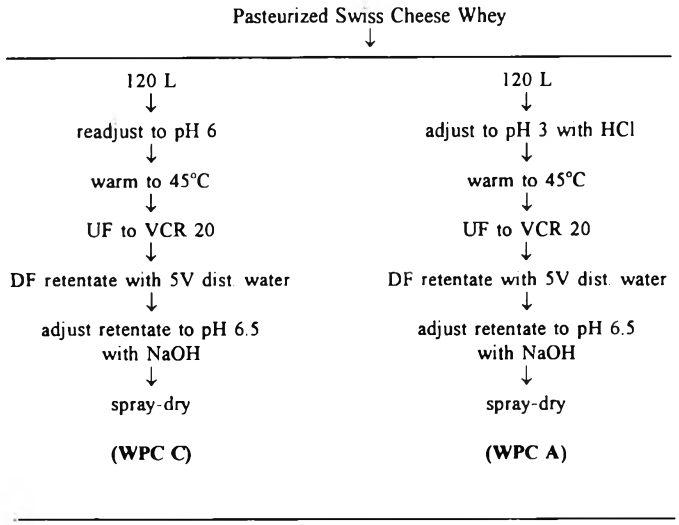

Fig. 1—Processing flow-chart for making WPC C and A.

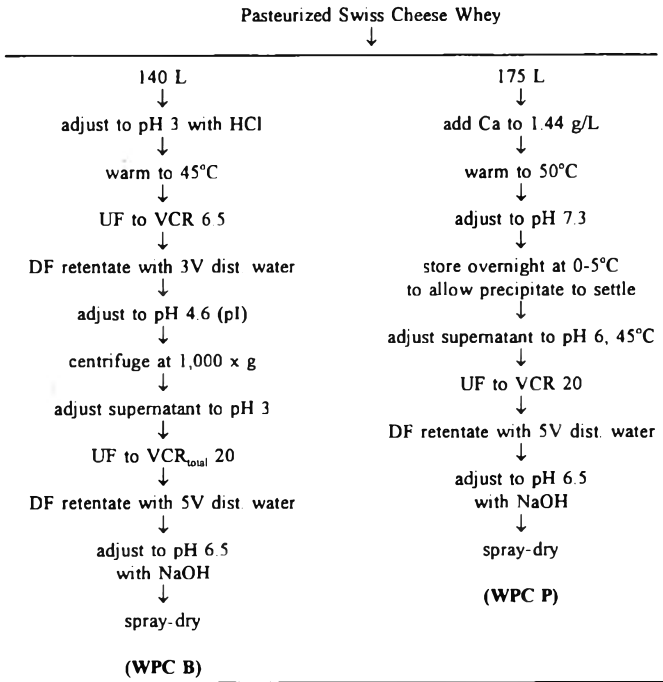

Fig. 2—Processing flow-chart for making WPC B and P.

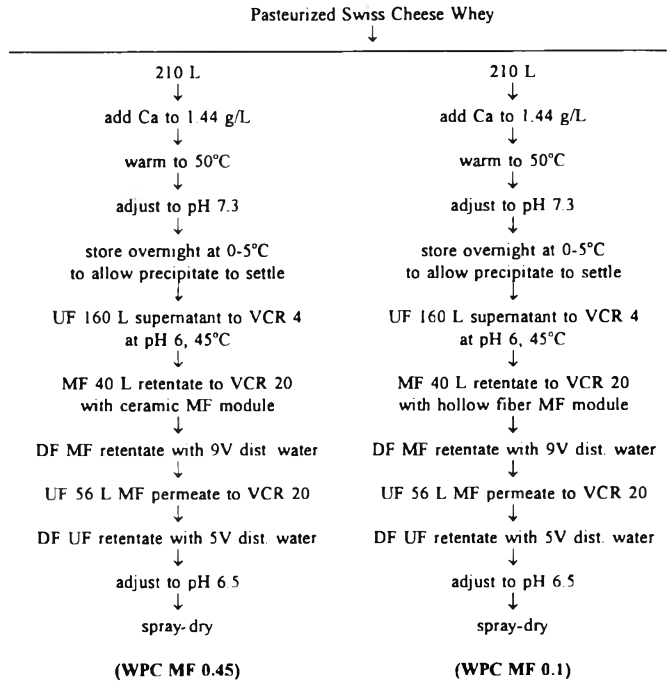

Fig. 3—Processing flow-chart for making WPC MF 0.45 and WPC MF 0.1.

the method of Fauquant et al. (1985) to remove PLPC and MF fractionated with 0.45-µm and 0.1-µm membranes, respectively (Fig. 3).

Volume concentration ratio (VCR) was computed as:

$$VCR = \frac{Volume_{feed}}{Volume_{retentate}}$$

Instant protein permeation ratios (PPR) were computed for 20 mL permeate aliquots as:

$$PPR = \frac{Protein\ concentration_{MF\ permeate}}{Protein\ concentration_{initial\ feed}}$$

Total protein recovery (%) was computed for the global MF and DF processes as:

$$\frac{Mean\ protein\ concentration_{pooled\ permeate} \times Volume_{permeate}}{Mean\ protein\ concentration_{initial\ feed} \times Volume_{feed}} \times 100$$

Analytical

Total solids of spray-dried WPC powder samples were determined by a procedure adapted from AOAC (1984). Triplicate samples were accurately weighed into separate aluminum dishes, dried in a convection oven for 16–18 hr at 100°C, cooled in a desiccator and reweighed. True protein content of WPC was determined in triplicate by the macro-Kjeldahl method according to Barbano et al. (1991), using a conversion factor of 6.38 (AOAC, 1984). Total lipid and phospholipid contents of WPC were determined by the method of Morr and Seo (1988). Total lipids were extracted with a mixture of methanol and chloroform (Bligh and Dyer, 1959) and extracts were fractionated on PrepSep-Si sep-pak columns (Fisher Scientific, Fairlawn, NJ) by the method of Juaneda and Rocquelin (1985). Total ash contents were determined by a procedure adapted from AOAC (1984). Triplicate 1g WPC samples were weighed accurately into separate porcelain crucibles, ignited in an electric muffle furnace at 600°C, cooled in a desiccator, and reweighed. Calcium determinations were made by atomic absorption spectroscopy of ashed samples using a 2-channel, double-beam IL 951 Video II atomic absorption spectrophotometer (Instrumentation Laboratory Inc., Wilmington, MA) equipped with microcomputer and screen readout by the procedure of Pollman (1991).

Protein composition

Size exclusion HPLC (SE-HPLC) was performed by the method of Morr (1987) with a TSK-3000 SW Spherogel column (Beckman Instruments, Inc., Palo Alto, CA), using a 0.1M, pH 6.0, phosphate buffer containing 0.1M sodium nitrate as eluant. The column was eluted isocratically at 0.5 mL/min with a 1050 HPLC system (Hewlett-Packard, Palo Alto, CA) equipped with an autosampler, a variable-wavelength UV detector set at 280 nm, and a Model HP 3396A integrator. Standard solutions of immunoglobulins (Ig), bovine serum albumin (BSA), β-lactoglobulin (β-Lg) and α-lactalbumin (α-La) from Sigma Chemical Company (St. Louis, MO) were used to determine retention times and protein concentrations.

SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was conducted according to Laemmli (1970) using Tris-HCl mini protean II ready gels, with a 4% acylamide stacking gel and a 12% acylamide resolving gel (Bio-Rad Laboratories, Hercules, CA). The gels were assembled in a Bio-Rad Mini-Protean II unit and protein bands were stained with Coomassie brilliant blue R-250. Low-molecular-weight standards from Bio-Rad Laboratories (Richmond, CA) were run simultaneously to determine protein molecular weight (MW). Minor proteins were tentatively

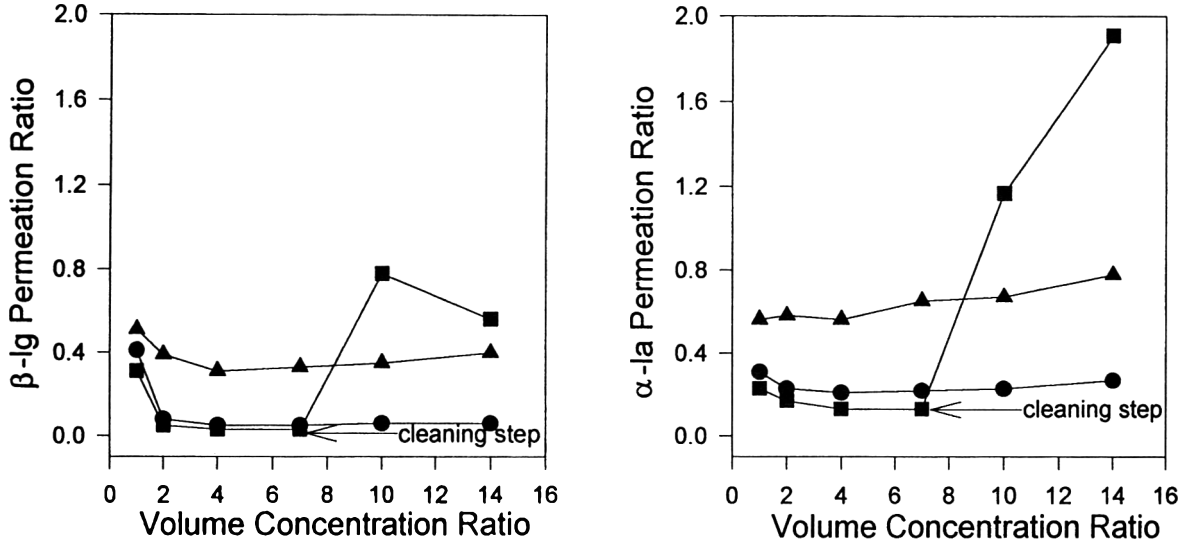

Fig. 4—MF-0.1 μm membrane permeation ratios of β-Lg and α-La as related to VCR for three MF processes: (1) ● One-step MF; (2) ■ Two-step MF; and (3) ▲ UF preconcentration to VCR 4-MF.

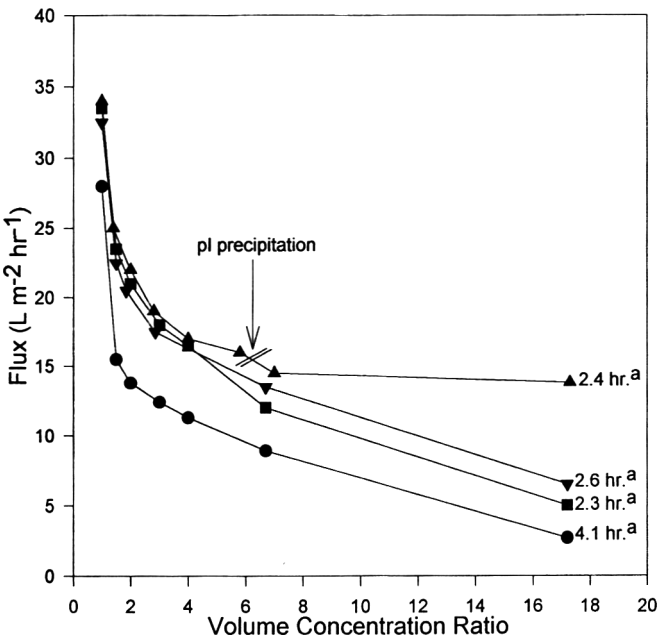

Fig. 5—UF membrane permeate flux as related to VCR for: (1) ● pH 6.0 whey, Process C; (2) ▼ pH 3.0 whey, Process A; (3) ■ chemically pretreated whey, Process P; and (4) ▲ pH 3.0 whey, Process B. [a]Total UF processing time.

identified according to Pallavicini et al. (1988) and Yoshida (1988). Video images of replicate electrophoresis gels were analyzed with a Tracor Northern image analysis computer (model TN8502, Tracor Northern, Middleton, WI) to estimate minor protein concentrations using intensity values ranging from 0 for white to 255 for black (Foster et al., 1992).

Statistical analysis

All data were analyzed by analysis of variance (ANOVA) of SAS Institute, Inc. (1988). Means were compared using the least significant difference test (Miller, 1985).

RESULTS & DISCUSSION

Preliminary experiment

A preliminary experiment was conducted to compare protein, i.e., α-La and β-Lg, permeation ratios by the 0.1-μm MF membrane from 3 different processes. Each of the 3 processes was conducted with 100 L of chemically pretreated whey: (1) a one-step MF process to VCR 14; (2) a two-step MF process to VCR 14, i.e., MF to VCR 7, clean the membrane, and MF to VCR_{total} 14; and (3) UF preconcentrate to VCR 4 and MF the UF retentate to VCR_{total} 4.

The UF preconcentration MF process (Fig. 4) resulted in higher β-Lg and α-La permeation ratios than the other two MF processes. The second step of the two-step MF process, after membrane cleaning, resulted in β-Lg permeation ratios of 0.78 and 0.56 at VCR 10 and 14, respectively. The α-La permeation ratios were 1.17 and 1.91 at VCR 10 and 14, respectively. These compare to initial β-Lg and α-La permeation ratios of 0.31 and 0.23 for step one of the process, respectively. Considering the complete processes, MF of UF preconcentrated whey resulted in higher permeation ratios for both β-Lg and α-La ($P < 0.05$). Total β-Lg recoveries for one-step MF, two-step MF, and UF preconcentration MF processes were 10, 13 and 35%, respectively, whereas total α-La recoveries for these processes were 26, 34 and 54%, respectively. The UF preconcentration MF process resulted in higher protein permeation ratios ($P < 0.05$), and, for this reason, that procedure was adopted for the remainder of the study.

Effect of pretreatment on UF flux

Initial UF membrane permeate flux values for the four WPC processes (C, A, B and P) were in the range of 28–34 L m^{-2} hr^{-1} (Fig. 5). Whey at pH 6.0 (Process C) exhibited the greatest membrane permeation flux reduction and, consequently, required a processing time of 4.1 hr compared to 2.3 (for P) and 2.6 (for A). Process B whey, which was preconcentrated to VCR 6.5 at pH 3 and adjusted to pH 4.6 to isoelectrically precipitate PLPC exhibited the highest final UF membrane flux of about 18 L m^{-2} hr^{-1}. Whey at pH 3.0 exhibited the next highest final UF membrane permeate flux of about 6 L m^{-2} hr^{-1}. These confirmed results of other researchers who stated that adjusting whey to pH 3 (Lee and Merson, 1976; Patocka and Jelen, 1987), and chemical pretreatment of whey (Maubois et al., 1987; Rinn et al., 1990) improve UF membrane permeate flux.

Comparison of permeate flux and protein permeation for two MF membrane types

Both the MF membranes displayed generally similar α-La and β-Lg permeation ratio profiles, except during initial stages (Fig. 6). A slight decrease in permeation ratios was observed for both

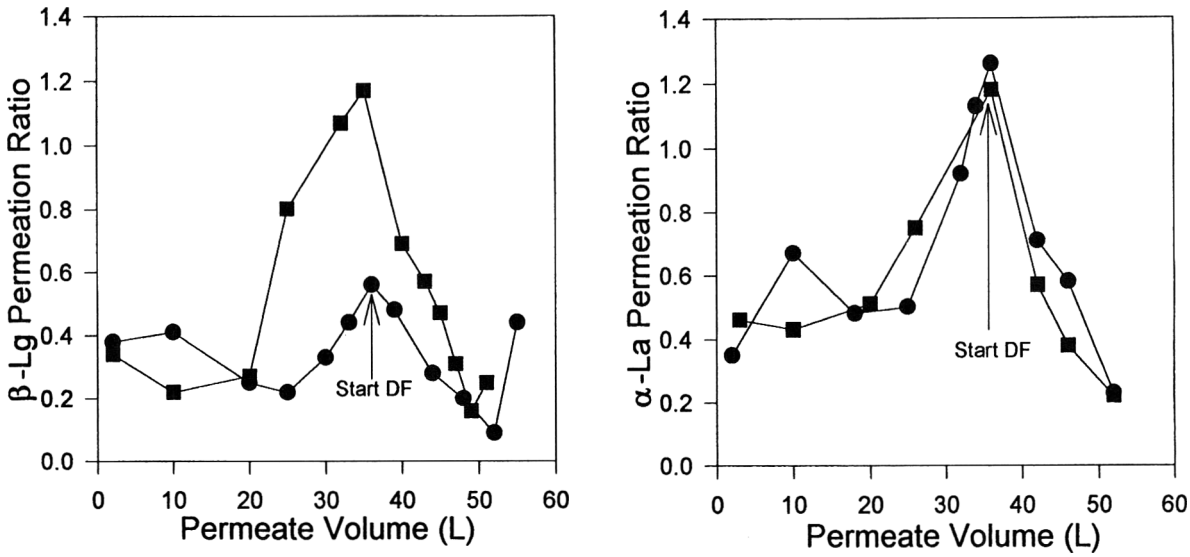

Fig. 6—MF membrane permeation ratios of β-Lg and α-La as related to permeate volume for making: (1) ■ WPC MF 0.45; and (2) ● WPC MF 0.1.

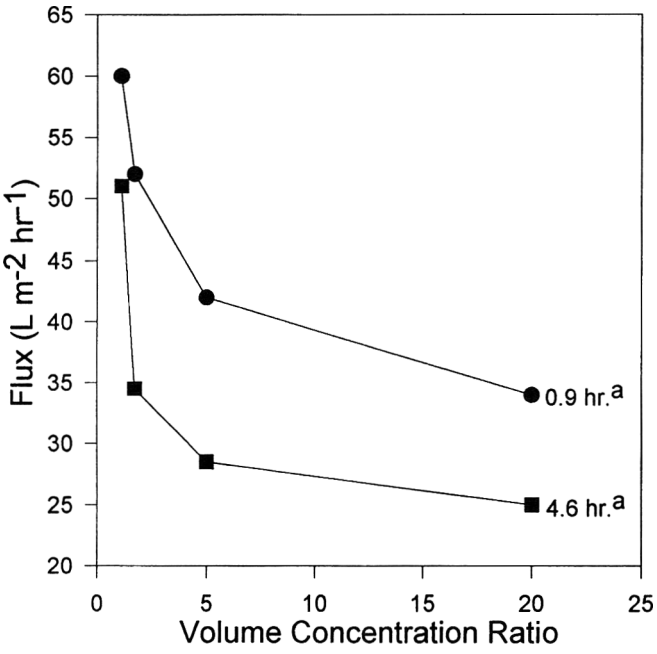

Fig. 7—MF membrane permeation flux during WPC processing: (1) ● 0.1 μm; and (2) ■ 0.45 μm. [a]MF processing time.

Table 1—WPC composition

	WPC products*					
	C	A	B	P	MF 0.1	MF 0.45
Total solids (%)	96.41[a]	97.66[a]	96.80[a]	96.65[a]	94.19[a]	96.60[a]
True protein (%)	75.1[b]	75.0[b]	81.9[a]	77.0[b]	73.9[b]	80.30[a]
Lipids (mg/g)	53.09[a]	43.15[b]	20.45[c]	38.76[b]	2.65[d]	5.20[d]
Phospholipids (mg/g)	5.60[a]	5.66[a]	1.49[bc]	6.08[a]	0.42[c]	2.79[b]
Ash (%)	2.69[c]	4.98[a]	4.48[b]	2.64[c]	3.00[c]	2.81[c]
Ca (mg/g)	2.78[b]	0.04[e]	0.06[e]	3.89[a]	1.71[c]	0.67[d]

* Data represent the means of three determinations of duplicated processes from six different processes.

[a-e] Means within the same row bearing different superscripts differ ($P<0.05$).

α-La and β-Lg during collection of the first 10L of permeate by MF 0.45, whereas MF 0.1 μm exhibited a pronounced increase in permeation ratio. About 20–30% of the total permeate is collected during this initial stage of the process, thus the observed differences in protein permeation ratio would be expected to affect total protein recovery. MF membrane permeation ratios for both α-La and β-Lg steadily increased after collection of about 20 L of permeate, reaching a maximum just before starting DF and steadily decreased during the remainder of the processes until 50 L of permeate was collected. Subsequent increases in permeation ratios for β-Lg for both MF membranes were observed. The MF 0.45 membrane gave consistently higher β-Lg permeation ratios than MF 0.1 throughout most of the process, whereas both membranes gave comparable α-La permeation ratios.

The 0.45-μm MF membrane provided a higher β-Lg recovery ($P < 0.05$) than the 0.1-μm membrane, whereas the MF 0.1 μm membrane provided higher α-La recovery than the 0.45-μm membrane ($P < 0.05$). β-Lg recovery in the permeate fraction of the 0.1- and 0.45-μm MF membranes were 51 and 86%, respectively, whereas recovery of α-La in the permeate fraction of these two membranes were 75 and 63%, respectively. The constant volume diafiltration step of the process recovered up to 15 and 28% α-La and β-Lg, respectively.

MF 0.1-μm membrane flux for pretreated, UF VCR 4 preconcentrated whey was higher than that for MF 0.45 μm (Fig. 7). This difference in membrane flux resulted in a 4-fold greater processing time for the 0.45-μm membrane than for the 0.1-μm membrane.

Chemical composition of WPC products

True protein contents ranged from about 74 to 77% for MF 0.1, A, C and P WPC to 80 to 82% for MF 0.45 and B WPC (Table 1). Total lipids of the WPC ranged from 2.65 to about 53 mg/g. WPC produced by MF of chemically pretreated whey, i.e., MF 0.1 and 0.45, contained the lowest total lipids. Comparison of WPC C and P indicated that chemical pretreatment, *per se*, was ineffective for removing total lipids from whey. The Breslau et al. (1975) delipidization procedure was more effective for removing total lipids than the thermocalcic pretreatment of Attebery (1971) ($P < 0.05$). WPC resulting from processes that included both chemical pretreatment and MF steps contained up to 95% less RWL than WPC C. Phospholipid contents of the various WPC products exhibited generally similar trends as total lipids. For example, WPC MF and B contained significantly lower concentrations of phospholipids than WPC C, A, and P ($P < 0.05$). Lipids/phospholipids ratios varied from 1.86 (MF 0.45) to 13.72 (B). We could not explain these differences.

Ash contents were generally similar for all WPC products. As expected, WPC A and B contained highest ash concentrations, due largely to the use of large quantities of NaOH to

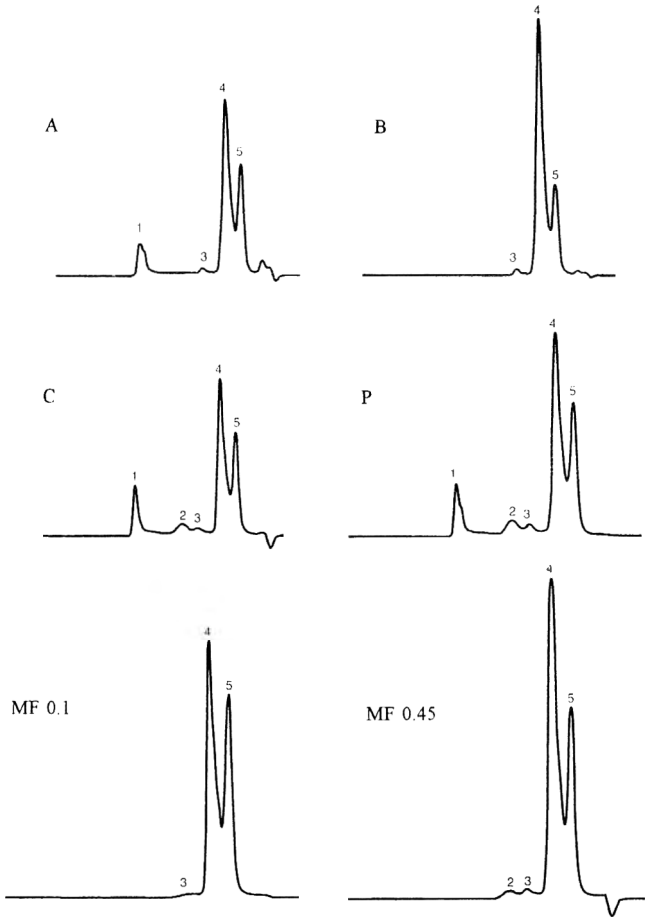

Fig. 8—Size exclusion HPLC patterns of WPC from the different processes. (1) Phospholipoproteins with retention time (RT) of 13.4 min; (2) Ig with RT of 19.1 min; (3) BSA with RT = 21.3 min; (4) β-Lg with RT = 24.2 min; and (5) α-La with RT = 26.6 min.

Table 2—Protein composition of WPC by size-exclusion HPLC

	WPC products*					
	C	A	B	P	MF 0.1	MF 0.45
Ig	6.6^{a}	0.8^{bc}	1.2^{bc}	6.7^{a}	0.3^{c}	1.7^{b}
BSA	5.0^{ab}	3.6^{abc}	3.5^{abc}	5.6^{a}	1.2^{d}	2.0^{cd}
β-Lg	74.5^{bc}	78.9^{ab}	83.8^{a}	72.1^{c}	78.9^{ab}	82.3^{a}
α-La	13.9^{bc}	16.7^{ab}	11.5^{c}	15.6^{b}	19.6^{a}	13.9^{bc}
Ratio α/β	0.19^{bc}	0.22^{ab}	0.14^{d}	0.22^{ab}	0.25^{a}	0.17^{cd}

* Data (Percent of total protein) represent the means of four determinations of duplicated processes from six different processes.

[a-d] Means within the same row bearing different superscripts differ ($P<0.05$)

neutralize their UF retentate fractions to pH 6.5 prior to spray-drying. WPC A and B, made by membrane processing at pH 3, both exhibited extremely low Ca concentrations (in the range 0.04–0.06 mg/g). Since the chemical pretreatment used for making WPC P and both MF WPC involved the addition of $CaCl_2$, higher Ca contents in those products were expected. Calcium concentrations showed differences in the following order: P > C > MF 0.1 > MF 0.45 ($P < 0.05$).

Protein composition of WPC products

SE HPLC results (Fig. 8, Table 2) for immunoglobulin contents of WPC C and P (6.6 and 6.7%, respectively) were similar to those reported by Rinn et al. (1990). All other WPC contained lower Ig concentrations, ranging from 1.7% (MF 0.45) to 0.3% (MF 0.1) ($P < 0.05$), due probably to removal of this large molecular weight, i.e., ≥ 146 kDa (Morr and Ha, 1993), protein by MF processing and by processing associated with the Breslau et al. (1975) method. BSA concentrations ranged from 3.5 to

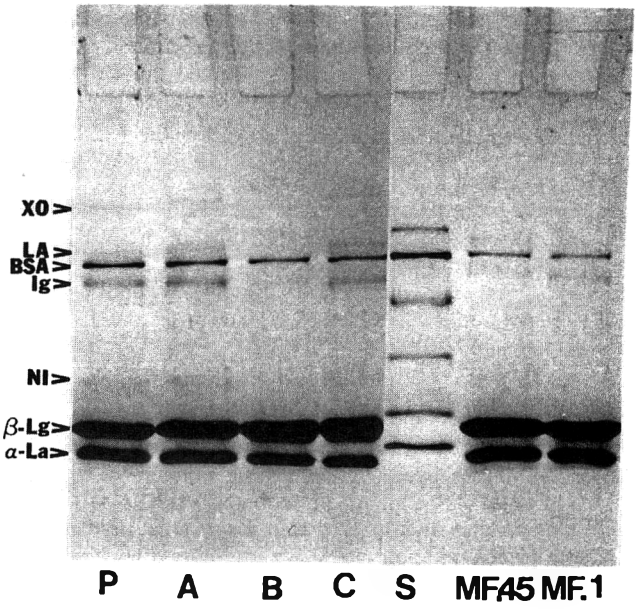

Fig. 9—SDS gel electrophoresis patterns of WPC proteins from six different processes: Xanthine oxidase (XO, 150 kDa), lactoperoxidase (LA, 89 kDa), BSA (66 kDa), IgG heavy chain (57 kDa), nonidentified protein (NI, 30 kDa), β-Lg (18 kDa), and α-La (14 kDa); S (standards).

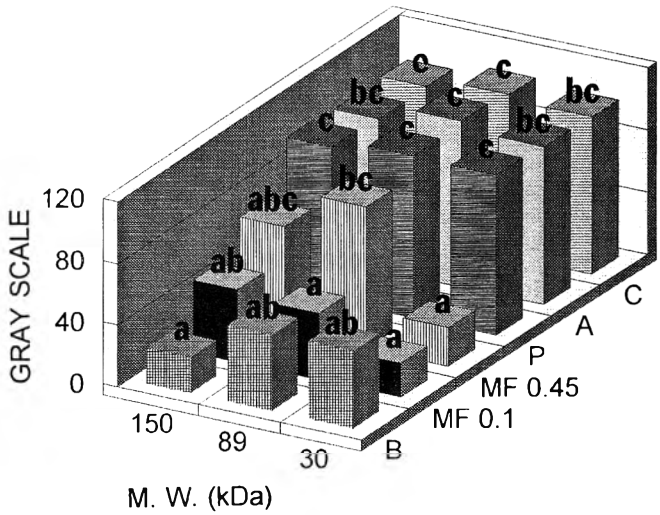

Fig. 10—Minor protein composition profile of WPC from seven different processes. [a-c]Means (n = 4) within the same molecular weight (MW) category bearing different letters differ in concentration ($P < 0.05$).

5.6 for all WPC except MF 0.1 and 0.45, due also to the likely removal of this large-molecular-weight (i.e., 66 kDa) protein during MF fractionation. On the other hand, thermocalcic pretreatment did not affect the concentration of either of these 2 whey proteins. α-La mean concentration values ranged from 11.5 to 19.6% and β-Lg values ranged from 72.1 to 82.3%. WPC B and MF 0.45 exhibited highest β-Lg levels while WPC P had the lowest β-Lg value. The highest α/β ratio was obtained for WPC MF 0.1 (0.25) and the lowest α/β ratio was for WPC B (0.14). Comparing WPC A and B for contents in β-Lg and α-La showed that pI precipitation resulted in increased β-Lg and decreased α-La. As expected, WPC MF 0.45 resulted in a lower content in α-La and a high content in β-Lg compared to WPC MF 0.1, in agreement with the better permeation ratios of α-La for process MF 0.1 and β-Lg for process MF 0.45 (Fig. 6).

SDS-PAGE patterns (Fig. 9) provided apparent confirmation of the relative concentrations of the 4 major whey proteins in each of the WPC products; however, these proteins were not quantified. Minor protein bands presumed to be xanthine oxidase (150 kDa), lactoperoxidase (89 kDa), and an unidentified 30 kDa protein were semi-quantified by image analysis (Fig. 10). Intensity values for the 150 kDa protein band were highest for WPC C and P and lowest for WPC B. WPC C, A and P contained the highest concentrations of 89 kDa protein, whereas WPC MF 0.1 had the least. Concentrations of the 30 kDa protein, in descending order, were in WPC P, A, C, B and the two MF WPC products. These minor proteins generally were in lower concentrations in WPC MF 0.1 and B ($P < 0.05$).

CONCLUSIONS

MICROFILTRATION in conjunction with chemical pretreatment was feasible to produce WPC containing as low as 0.2% lipids. Pretreatment involving pI precipitation of PLPC was more efficient than thermocalcic aggregation for residual lipid removal. However, pI precipitation was successful only after UF preconcentration to VCR 6.5. Protein permeation and flux results showed that optimization of MF processing is needed.

Further work will assess improvements in functional properties and flavor stability of these delipidized WPC products and assess potential uses of MF retentate fractions with higher Ig and BSA.

REFERENCES

American Dairy Products Institute 1993. Whey products: A survey of utilization and product trends. Chicago, IL.
AOAC 1984. *Official Methods of Analysis*, 14th ed. Association of Official Analytical Chemists, Washington, DC.
Attebery, J.M. 1971. Removing lipid material from whey. U.S. Patent # 3 560 219.
Barbano, D.M., Lynch, J.M., and Fleming, R. 1991. Direct and indirect determination of true protein content of milk by Kjeldahl analysis: Collaborative study. J. Assoc. Off. Anal. Chem. 74: 281–288.
Bligh, E.G. and Dyer, W.J. 1959. A rapid method of total lipid extraction and purification. Can. J. Biochem. Physiol. 37: 911–917.
Breslau, B.R., Goulet, J., and Cross, R.A. 1975. Production of a crystal clear, bland tasting protein solution from cheese whey. Cultured Dairy Products J. 10(4): 13–14.
De Wit, J.N., Klarenbeek, G., and Adamse, M. 1986. Evaluation of functional properties of whey protein concentrates and whey protein isolates. 2 Effects of processing history and composition. Neth. Milk Dairy J. 40: 41–56.
Fauquant, J., Vieco, E., Brule, G., and Maubois, J.-L. 1985. Clarification des lactosérums doux par agrégation thermocalcique de la matière grasse résiduelle. Le Lait 65: 1–20.
Foster, D.N., Min, B., Foster, L.K., Stoflet, E.S., Sun, S., Getz, M.J., and Strauch, A.R. 1992. Positive and negative *cis*-acting regulatory elements mediate expression of the mouse vascular smooth muscle α-actin gene. J. Biol. Chem. 267: 11995–12003.
Hanemaaijer, J.H. 1985. Microfiltration in whey processing. Desalination 53: 143–155.
Hiddink, J., de Boer, R., and Nooy, P.F.C. 1981. Effect of various pretreatments on the ultrafiltration of sweet cheese whey at about 55°C. Milchwissenschaft 36: 657–663.
Juaneda, P. and Rocquelin, G. 1985. Rapid and convenient separation of phospholipids and non-phosphorus lipids from rat heart using the silica cartridges. Lipids 20: 40–41.
Kim, S.H., Morr, C.V., Seo, A., and Surak, J.G. 1989. Effect of whey pretreatment on composition and functional properties of whey protein concentrate. J. Food Sci. 54: 25–29.
Knopp, T.K. 1992. Lipoprotein removal from cheese whey by cross-flow microfiltration. M.S. Thesis, The Ohio State University, Columbus.
Kuo, K.P. and Cheryan, M. 1983. Ultrafiltration of acid whey in a spiral-wound unit: Effect of operating parameters on membrane fouling. J. Food Sci. 48: 1113–1118.
Laemmli, U.K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227: 680–685.
Laye, I. 1993. Effect of processing on the composition of whey protein concentrate and subsequently on the development of volatile organic compounds during storage. Ph.D. Dissertation, The Ohio State University, Columbus.
Laye, I., Karleskind, D., and Morr, C.V. 1994. Chemical and volatile organic compounds composition of whey protein concentrate. Proceedings of the 8th International Flavor Conference, Cos, Greece, July 6–8.
Lee, D.N. and Merson, R.L. 1976. Chemical treatments of cottage cheese whey to reduce fouling of ultrafiltration membranes. J. Food Sci. 41: 778–786.
Lehmann, H. and Wasen, D. 1990. Method of dephosphilidating whey. U.S. Patent # 4 897 279.
Maubois, J-L., Pierre, A., Fauquant, J., and Piot, M. 1987. Industrial fractionation of main whey proteins. In *Bull. 212, Trends in Whey Utilization*, p. 154–159. Int. Dairy Fed., Brussels, Belgium.
Merin, U., Gordin, S., and Tanny, G.B. 1983. Microfiltration of sweet cheese whey. N. Z. J. Dairy Sci. Technol. 18: 153–160.
Miller, R. 1985. Multiple comparisons. In *Encyclopedia of Statistical Sciences*, S. Kotz and N.L. Johnson (Ed.), 5: 679. John Wiley & Sons, Inc., New York.
Morr, C.V. 1987. Effect of HTST pasteurization of milk, cheese whey and cheese whey UF retentate upon the composition, physicochemical and functional properties of whey protein concentrates. J. Food Sci. 52: 312–317.
Morr, C.V. 1992. Improving the texture and functionality of whey protein concentrate. Food Technol. 46(1): 110–113.
Morr, C.V. and Foegeding, E.A. 1990. Composition and functionality of commercial whey and milk protein concentrates and isolates: a status report. Food Technol. 44(4): 100–112.
Morr, C.V. and Ha, E.Y.W. 1991. Off-flavors of whey protein concentrates: a literature review. Int. Dairy J. 1: 1–11.
Morr, C.V. and Ha, E.Y.W. 1993. Whey protein concentrates and isolates: processing and functional properties. CRC Crit. Rev. Food Sci. Nutr. 33: 431–476.
Morr, C.V. and Seo, A. 1988. Unpublished. Clemson University, SC.
Pallavicini, C., Pagliarini, E., and Peri, C. 1988. Effect of a proteolytic enzyme preparation (alcalase) on whey proteins. J. Dairy Sci. 71: 887–892.
Patocka, J. and Jelen, P. 1987. Calcium chelation and other pretreatments for flux improvement in ultrafiltration of cottage cheese whey. J. Food Sci. 52: 1241–1244.
Pierre, A. and Fauquant, J. 1986. Principes pour un procédé industriel de fractionnement des protéines du lactosérum. Le Lait 66: 405–419.
Pollman, R.M. 1991. Atomic absorption spectrophotometric determination of calcium and magnesium and colorimetric determination of phosphorus in cheese: collaborative study. J. Assoc. Off. Anal. Chem. 74: 27–31.
Rinn, J.-C., Morr, C.V., Seo, A., and Surak, J.G. 1990. Evaluation of nine semi-pilot-scale whey pretreatment modifications for producing whey protein concentrate. J. Food Sci. 55: 510–515.
SAS Institute, Inc. 1988. *SAS/STAT User's Guide. Release 6.03 Edition*. Statistical Analysis System Institute, Inc., Cary, NC.
Yoshida, S. 1988. Isolation of some minor milk proteins, distributed in acid whey from approximately 100,000 to 250,000 daltons of particle size. J. Dairy Sci. 71: 1–9.

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Zymogen Activation in Pancreatic Endoproteolytic Preparations and Influence on Some Whey Protein Hydrolysate Characteristics

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ABSTRACT

A proteolytic preparation from porcine pancreas was isolated. Trypsin, chymotrypsin and elastase were characterized and their time-dependent stability at 37°C was studied. The supernatant of a 30% (w/v) saturated ammonium sulfate precipitation of a pancreatic extract (30S) was developed to pilot-scale level. The influence of zymogen activation time on molecular characteristics of whey protein hydrolysates produced by 30S and the commercial pancreatic preparation Corolase PP were compared. Amino acid analysis and gel permeation chromatography were used to characterize lactalbumin hydrolysates produced. Physicochemical characteristics of whey protein hydrolysates could be altered by manipulation of zymogen activation conditions in pancreatic proteinase preparations to be used during subsequent protein hydrolysis.

Key Words: whey protein, hydrolysates, endoprotease, zymogen activation

INTRODUCTION

ENZYMATIC MODIFICATION OF PROTEINS using selected proteolytic enzyme preparations to split specific peptide bonds is widely used in the food industry (Godfrey and Reichelt, 1983; Adler-Nissen, 1986; Fox, 1991; Arai and Fujimaki, 1991). Whey proteins enzymatically hydrolyzed with trypsin and chymotrypsin show better solubility and *in vitro* digestibility than their chemically treated counterparts (Lakkis and Villota, 1992). The choice of substrate, protease employed and degree to which the protein is hydrolyzed affect physicochemical properties of resulting hydrolysates. By controlling reaction conditions during enzymatic hydrolysis of milk proteins, hydrolysates with different solubility and emulsifying characteristics (Turgeon et al., 1992), foaming properties (Kuehler and Stine, 1974) or taste (Murray and Baker, 1952; Poulsen, 1987; Vegarud and Langsrud, 1989) have been produced.

The degree to which milk proteins are hydrolyzed depends on the intended use for the hydrolysate. Low degrees of hydrolysis are desirable for maintaining functional properties. Extensive hydrolysis may be necessary when the hydrolysate is to be used for specific purposes such as chemically defined or hypoallergenic infant formula (Merritt et al., 1990; Thibault, 1991). Mixtures of proteases which have complementary specificities may be used to attain a required degree of hydrolysis. The number and ratio of proteolytic activities is critical to the final hydrolysate characteristics. The proportions of different proteinases, such as trypsin to chymotrypsin and the presence or absence of exopeptidases, such as carboxypeptidase B, may be important in the production of hypoallergenic hydrolysates (Thibault, 1991), or those with reduced bitterness (Fullbrook et al., 1987; Plainer and Sprossler, 1990).

Little information has been published which relates critical ratios of proteinase/peptidase activity of commercial proteases used for hydrolysis of milk proteins to physicochemical characteristics of the final hydrolysate. Many groups have devised processes for production of milk protein hydrolysates using commercial pancreatic proteases, i.e., for clinical nutrition (Grimble and Silk, 1989; Maubois and Léonil, 1989), hypoallergenic and special dietetic products (Jost et al., 1988; Asselin et al., 1988, 1989; Thibault, 1991). However, the choice of enzyme preparation has usually been based on empirical screening or random selection.

Published methods for purification of pancreatic proteinases have mainly focused on isolation of individual enzymes with elimination of other activities (Bergmann and Fruton, 1941; Northrop et al., 1948; Lewis et al., 1959; Balls, 1965). Purification, physical and chemical properties of both the zymogen, and active forms of pancreatic proteinases have been reviewed, i.e., trypsin (Northrop et al., 1948; Desnuelle and Rovery, 1961; Hakim et al., 1962; Vestling et al., 1990), chymotrypsin (Wilcox, 1970), elastase (Hartley and Shotton, 1971; Gertler et al., 1977). Limited studies have been published on the enrichment of a mixture of pancreatic enzymes where the activity of all enzymes was retained, or where a desired ratio of proteinases was required to mimic the *in vivo* digestive system. Prior to any pancreatic protease development, factors governing the specific activity of the proteolytic enzymes must be considered. Specific activities can be influenced by many factors. These include activation of one enzyme by another (Desnuelle and Rovery, 1961); autolysis/degradation (Vestling et al., 1990); presence or absence of metal ions; hormonal activators in the tissue source (Padfield and Case, 1987), reverse feed-back mechanisms in the diet (Dagorn and Lahaie, 1981) and species differences (Marchis-Mouren, 1965).

Our study considered stability (Vestling et al., 1990; Vithayathil et al., 1961; Buck et al., 1962; Hakim et al., 1962) and activation of the zymogen forms of proteolytic enzymes of porcine pancreas (Desnuelle and Rovery, 1961). This tissue was chosen for this study as it is known that porcine trypsin has better stability with respect to heat, influence of metal ions and pH changes (Buck et al., 1962). Furthermore, porcine pancreatic proteases have similarities with pancreatic proteases of human origin (Clemente et al., 1972; Gertler et al., 1977; Largmann et al., 1976).

Our objective was to develop methods for isolation and activation of porcine pancreatic proteolytic preparations. These preparations were compared with commercial proteases. Effects of the enzyme isolation and activation conditions on the development and associated physicochemical properties of whey protein hydrolysates were compared.

MATERIALS & METHODS

Materials

Commercial pancreatic proteases were gifts from manufacturers. Corolase PP (porcine) was supplied by Röhm GmbH, Darmstadt, Germany. PTN 3.0S (porcine) and PEM 2500S (porcine) were supplied by Novo Nordisk A/S, Bagsvaerd, Denmark. Bovine trypsin (Type XII) was obtained from Sigma Chemical Co., Poole, Dorset, UK. Amino-methyl-coumarin (AMC) and the fluorogenic substrates for elastase, chymotrypsin

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and trypsin, were acetyl-ala-ala-pro-ala-AMC, N-succinyl-leu-leu-val-tyr-AMC and N-benzoyl-L-arg-AMC, respectively, obtained from Bachem, Bubendorf, Switzerland. Lactalbumin (Alatal-560) was from New Zealand Dairy Board, Wellington, New Zealand. It is an insoluble heat-denatured whey protein and its manufacture has been described (Robinson et al., 1976; Mulvihill, 1992). The standard proteins and peptides for FPLC were from Sigma and were as follows: Blue dextran (2,000,000 Da), Immunoglobulin G (150,000), bovine serum albumin (67,500), β-lactoglobulin (36,000), α-lactalbumin (14,700), insulin chain B (3,494), DL-tryptophan (204), L-tyrosine (181), L-arginine (174). Reagents and standards for amino acid analysis were obtained from Beckman Instruments, High Wycombe, U.K. All other reagents were of analytical grade unless otherwise specified.

Preparation of pancreatic enzyme extracts

Extraction and ammonium sulfate fractionation. Initial steps in the method of Kunitz, as outlined by Laskowski (1956), were used and several modifications were made to account for scale-up. Fresh pancreas (5.25 kg) was from Cappoquin Meats Ltd. (Pork and Bacon Processors, Cappoquin, Co. Waterford) which was stored cold during transit to the laboratory and was then immersed in ice-cold 0.125M H_2SO_4. Fat was trimmed from pancreatic tissue, which was chopped and then macerated in a Waring blender for 5 sec. The macerated tissue was suspended in 0.125M H_2SO_4 (1:1 volume ratio of tissue to acid solution). Macerated tissue (≈5L Sample A) was obtained and this was stirred at 4°C overnight at 130 rpm in an equal volume of cold 0.125M H_2SO_4. The suspension (10L) was filtered through one layer of synthetic cheese cloth and the filtrate (5.2 L) was collected. Residual tissue was further macerated, using a large-scale blender, for 3 min and was again stirred overnight in an equal volume of 0.125M H_2SO_4. The suspension was centrifuged at 1900 × *g* (Mistral 6000, MSE Scientific Instruments, Crawley, West Sussex, U.K.) for 10 min at 0°C and the supernatant was combined with the previous filtrate to give a total extract volume of 10.95 L (Sample B). Several ammonium sulfate precipitations were carried out on 100-mL aliquots of this filtrate for initial studies. Saturated ammonium sulfate (SAS) solution was added to Sample B at 4°C from a separating funnel at a flow rate of 40 mL/min, with continuous stirring. The mixture was allowed to stand for 30 min and then centrifuged for 20 min at 10,000 × *g* at 0°C. The supernatant of the SAS fractionation was designated S (for example, 30S refers to the supernatant of the 30% ammonium sulfate precipitation of Sample B).

Desalting and concentration of protein in 30S preparation. In a larger-scale experiment the supernatant (1000 mL) from the 30% ammonium sulfate precipitation (30S) was vacuum-filtered on a Büchner funnel through Whatman no. 4 and no. 1 filter papers, consecutively, to remove suspended solids. An Amicon® (CH_2A Concentrator, Amicon Ltd., Stonehouse, Gloucestershire, U.K.) hollow-fiber ultrafiltration system was then used to desalt and concentrate the protein. Inlet pressure was maintained at ≈0.7 kg/cm² and the pump speed setting was maintained between 4–5. An Amicon® hollow-fiber membrane with a molecular weight cut-off of 30,000 Daltons and surface area of 0.06 m² was used (HIP 20–30 Serial no. 0032–1A120). A constant permeate flow rate of ≈7 mL/min was maintained during continuous diafiltration with 2.5 L distilled H_2O before the retentate was reduced to 200 mL (i.e., volume concentration reduction = 5). Samples from the permeate and retentate streams (≈5 mL) were removed at intervals and assayed for trypsin activity. The degree of diafiltration was monitored using continuous conductivity measurements (Philips PW9509 digital conductivity meter, Pye Unicam Ltd., Cambridge, U.K.).

Activation of zymogens

Initial experiments were carried out on Sample B and 30S to determine the activation/deactivation rate of principal endoproteinases. These fractions were buffered with 0.1 M Tris-HCl, pH 7.0, containing 100 mM $CaCl_2$ taking one volume protein solution and mixing with 9 volumes of Tris buffer. The protein suspension was adjusted to pH 7.0 and further diluted 1.45-fold using Tris buffer. Exogenous trypsin, Sigma type XII (1 mg/mL), was then added at 2% (v/v). A negative control was also used (i.e., sample without added trypsin). Subsamples (300 µL) of both extract fractions were taken at 1-hr intervals, frozen immediately and later assayed for trypsin, chymotrypsin and elastase using a modification of the standard fluorogenic assay (Zimmerman et al., 1977). To determine the effects of ammonium sulfate on elastase activity, a sample of 30S was desalted using a PD-10 Sephadex G25 column (Pharmacia LKB, Biotechnology Ltd., Uppsala, Sweden). Further studies were also carried out on desalted, concentrated 30S samples. Aliquots of these were activated, as above, for different times at 37°C (4, 16, 20, 28, and 44 hr), prior to freeze-drying. The enzymatic activity of trypsin, chymotrypsin and elastase was determined in 10 mg/mL solutions of each freeze-dried sample for different activation times. A concentrated desalted 30S sample was activated for 4 hr in the presence and absence of calcium chloride (30Snc) to determine the effects of Ca^{2+} ions on the activation/de-activation of the endoproteinases. Freeze-dried samples were stored in plastic containers at −20°C.

Pretreatment of Corolase PP preparation

Before hydrolysis of lactalbumin with Corolase PP, aliquots of this preparation (10 mg powder/mL) were incubated at 37°C for different time intervals (4, 16, 28 and 44 hr).

Quantification of enzyme activity

Specific activity. Enzyme activities were assayed using a modification of the standard fluorogenic assay (Zimmerman et al., 1977). Aqueous solutions of commercial proteases and freeze-dried 30S (10 mg powder/mL) in addition to pellets (redissolved to 100 mL) and supernatants (brought to 250 mL) from different SAS precipitations were centrifuged at 13,000 rpm (Microcentaur, MSE Scientific Instruments, West Sussex, U.K.) for 10 min. Twenty µL of supernatant was added to 980 µL of 0.01M Tris-HCl buffer, pH 7.0, containing 0.02 mM specific substrate. The substrate-enzyme mixture was incubated at 37°C for 1 hr. The reaction was stopped by the addition of 1 mL of 1.5M acetic acid and fluorescence was measured on a Perkin Elmer 1000 Spectrofluorimeter (Beaconsfield, Bucks, U.K.) at excitation and emission wavelengths of 360 nm and 440 nm, respectively. Activity units were defined as µmoles AMC/min/mg protein or µmoles AMC/min/total volume in each fraction. All enzymatic analyses were carried out in duplicate.

Total proteolytic activity. We used a modification of a previous method (Garcia de Fernando and Fox, 1991). Ten mL of 0.4% (w/v) azocasein in 0.02 M Tris/HCl buffer pH 8.0 was added to 1 mL enzyme solutions (0.01–1.00 mg/mL, i.e., sufficient to establish a linear activity response) and incubated at 50°C for 15 min. The reaction was stopped by adding 2 mL 12% (w/v) trichloroacetic acid (TCA) to an equal volume of reaction mixture. The mixture was filtered through Whatman no.40 filter paper and the absorbance at 440 nm of the supernatant was measured using a Cary 1/3 UV-VIS spectrometer (Varian Ltd, Mulgrave, Victoria, Australia). One unit of activity was defined as that amount of activity which gave a change of 1 absorbance unit at 440 nm/min/mg of enzyme preparation. For the ammonium sulfate precipitation experiments, endoproteolytic activity in the pellet and supernatants was expressed as a percentage of total activity in the starting material, i.e., Sample B.

Preparation of hydrolysates

A 25-mL solution of lactalbumin, 8% (w/v) protein, was hydrolyzed with individual protease preparations at 50°C, pH 8.0, for 240 min. The pH was maintained constant by continuous addition of 0.5M NaOH using a pH-stat (Metrohm Ltd, Herisau, Switzerland). The degree of hydrolysis (DH, %), defined as the percentage of peptide bonds cleaved, was calculated from the volume and molarity of NaOH used to maintain constant pH (Adler-Nissen, 1986). Following hydrolysis, proteases were inactivated by heating at 80°C for 30 min. Hydrolysates were then cooled and stored at −20°C for further analysis. The DH was calculated as follows:

$$DH\ (\%) = B \times N_b \times 1/\alpha \times 1/MP \times 1/h_{tot} \times 100$$

where B = volume of NaOH consumed (mL); N_b = normality of NaOH; α = average degree of dissociation of the α-NH_2 groups at pH 8.0 and 50°C; MP = mass of protein (g); h_{tot} = total number of peptide bonds in the protein substrate (m eqv/g protein). The h_{tot} value, 8.8 meqv/g for whey protein, and values of 1/α for various pH-temperature combinations were those given by Adler-Nissen (1986). The enzyme-to-substrate ratio (E:S) for lactalbumin hydrolysis experiments was calculated on the basis of (1) weight of enzyme preparation to weight of protein in the substrate, (2) total proteolytic activity units in the enzymatic preparation to weight of protein in the substrate, (3) tryptic activity (µmoles AMC/min/mg protein) in enzyme preparation to weight of protein in the substrate, or (4) protein in enzyme preparation to weight of protein in the substrate (Table 3).

Table 1—Activity[a] of trypsin, chymotrypsin and elastase in supernatants of ammonium sulfate purification fractions, expressed as percentage of total activity in the starting material (Sample B)

Purification fractions	Total activity (%)		
	Trypsin	Chymotrypsin	Elastase
Sample B	100.0	100.0	100.0
20S	–[b]	70.3	66.7
30S	95.0	33.7	37.8
30S (desalted)	—	—	37.6
35S	87.7	20.0	41.7
40S	73.6	19.7	0.0
30P[c]	—	—	47.4

[a] using fluorogenic assays as described in Materials and Methods section.
[b] not determined
[c] P refers to pellet fraction.

Table 2—Total proteolytic activity, final DH (after 240 min), and E:S ratio for lactalbumin hydrolysis experiments using commercial pancreatic preparations, pretreated Corolase PP and pancreatic extracts after different activation times

Proteolytic samples	Total proteolytic activity (units/min/mg prepn)	Activation time (hr)	Pretreatment time (hr)	E:S ratio[a]	Final DH (%)
PTN 3.0S	0.16	—	0	*[c]	8.83
PEM 2500S	0.90	—	0	*	8.57
Corolase PP	0.15	—	0	*	13.83
Corolase PP	—	—	4	0.02	13.11
Corolase PP	—	—	16	0.02	10.73
Corolase PP	—	—	28	0.02	9.82
Corolase PP	—	—	44	0.02	6.08
Pancreatic extracts					
Sample B	—	4	0	0.20	4.09
30Snc[b]	—	4	0	0.02	6.31
30S	—	4	0	0.02	5.81
30S	—	16	0	0.02	8.44
30S	—	28	0	0.02	7.47
30S	—	44	0	0.02	7.61

[a] E:S ratio was calculated on the basis of weight of preparation to protein in the substrate.
[b] 30S nc - preparation activated in the absence of calcium ions.
[c] E:S ratio was on the basis of total proteolytic activity units to protein in the substrate (i.e. 0.16 Azocasein units/g protein).

Characterization of hydrolysates

All protein, molecular size distribution and amino acid analyses were carried out in duplicate.

Protein (nitrogen) determination. Total protein (N × 6.25) of pancreatic preparations and of whey protein (N × 6.38) was determined by the micro-Kjeldahl method (AOAC, 1980).

Molecular size distribution of peptides in lactalbumin hydrolysates. A fast protein liquid chromatography system (FPLC) fitted with a Superose 12 gel permeation column (Pharmacia LKB Biotechnology Ltd., Uppsala, Sweden) was used to monitor the size distribution of peptides in lactalbumin hydrolysates. The column was eluted at 1 mL/min using 0.1M Tris-HCl buffer, pH 7.0, containing 0.1 M NaCl and 10% (v/v) methanol. Hydrolysates were diluted in elution buffer to 0.25% (w/v) protein, filtered through a Whatman 0.45 µm PS syringe filter and 100 µL was applied to the column in duplicate. Eluate was continually monitored at 214 or 280 nm using two single-path monitors (UV-1, Pharmacia) and data were recorded using a Minichrom© data handling system (VG, Data Systems, Altrincham, Cheshire, U.K.). A molecular weight calibration curve was prepared from the average retention volumes (Rv) of standard proteins and peptides.

Free amino acid analysis of hydrolysates. Hydrolysates were deproteinized by mixing equal volumes of 24% (w/v) TCA with sample and holding at room temperature for 30 min before centrifuging at 13,000 rpm (Microcentaur, MSE Scientific Instruments, Crawley, West Sussex, U.K.) for 10 min. Supernatants were removed and diluted with 0.2 M sodium citrate buffer, pH 2.2, to give ≈25 nmoles of each amino acid residue per 50 µL injection volume. Amino acids were analyzed on a 12 cm × 4 mm cation exchange column (Na+ form) using a Beckman 6300 amino acid analyzer (Beckman Instruments Ltd, High Wycombe, U.K.). Standard amino acids were used to calibrate the analyzer (see Materials).

RESULTS & DISCUSSION

ALTHOUGH PORCINE TRYPSIN is more thermostable than bovine trypsin (Lazdunski and Delaage, 1965), both are subject to autolysis. Porcine trypsin is denatured at 55°C, but it has been shown that, in the presence of whey protein as substrate, the enzyme retained >70% activity after 1 hr at 55°C (Buck et al., 1962; Jost et al., 1977). Trypsin is important, not only for its intrinsic activity but also in its activation of the other zymogens in the pancreas (Desnuelle and Rovery, 1961; Peanasky et al., 1969) and control of its activation can determine the extent of hydrolysis by pancreatic proteases. Chymotrypsin and elastase can also act as substrates for themselves and for each other. In addition, each pancreatic proteinase can again have different active forms which relates to the activation procedure employed (Wilcox, 1970; Martínez et al., 1981; Puigserver et al., 1986; Vestling et al., 1990; Guash et al., 1992). With a selected ratio of endoproteolytic activities during the activation process, in the presence of substrate (i.e., whey protein) such activities can be stabilized as the protein substrate will act as a protective agent (Jost et al., 1977).

Ammonium sulfate fractionation of pancreatic endoproteinases

At 35% SAS almost 90% trypsin, 20% chymotrypsin and >40% elastase original activities remained in solution (Table 1). Preliminary experiments showed that only proteolytic solutions with conductivity values less than that corresponding to a 1% ammonium sulfate solution would freeze-dry efficiently. The recovery of freeze-dried 30S proteolytic preparation was ~ 84g/kg pancreatic tissue. Trypsin activity was used as an index of activation. When the 30S preparation (10 mg/mL powder) was activated for 4 hr, it had 24 times less trypsin activity (µmoles/min/mg protein) than the commercial preparation PTN 3.0S (50.4 µmoles/min/mg protein).

Activation of pancreatic zymogens

The stability of trypsin, chymotrypsin and elastase, in addition to possible exopeptidase activities, appeared to be dependent on the duration of the activation procedure and factors such as storage conditions of the active product. Storage of the zymogen form of any of the proteolytic mixtures in 0.01M Tris-HCl buffer, pH 7.0, containing 100 mM $CaCl_2$ for 2 days at 4°C resulted in an increase in trypsin and chymotrypsin activity. However, after incubation at 4°C over 5–6 days in the same buffer, a decrease in activity of these enzymes was observed. Activation of both trypsinogen and chymotrypsinogen appeared to be enhanced following freezing and thawing at −20°C. Possibly freeze-thaw action may disrupt cells/granules resulting in release of zymogen form which is then activated or, alternatively, freezing may favor the formation of a specific optical rotatory form which would speed up the activation process (Walsh, 1970; Keil, 1971). We observed that holding extract fraction solutions at 37°C for 24 hr resulted in decreased activity for elastase and chymotrypsin.

In our study the activation of porcine proteolytic preparations was not dependent on the provision of Ca ions. The activation effect of Ca is less pronounced in porcine trypsin then in bovine trypsin (Lazdunski and Delaage, 1965; Abita et al., 1969). Interestingly, 30S preparation activated in the absence of Ca (30Snc) had a slightly higher DH value at 240 min than the sample activated with these cations (Table 2), though the lactalbumin hydrolysis curves were similar (Fig. 1). The 30Snc also produced a hydrolysate with more peptides in the <570 Da molecular weight range. Though trypsin activity levels in both preparations were the same, inclusion of Ca ions in the activation process may influence the formation of certain types of active enzyme products (Abita et al., 1969; Smith and Shaw, 1969; Vestling et al., 1990).

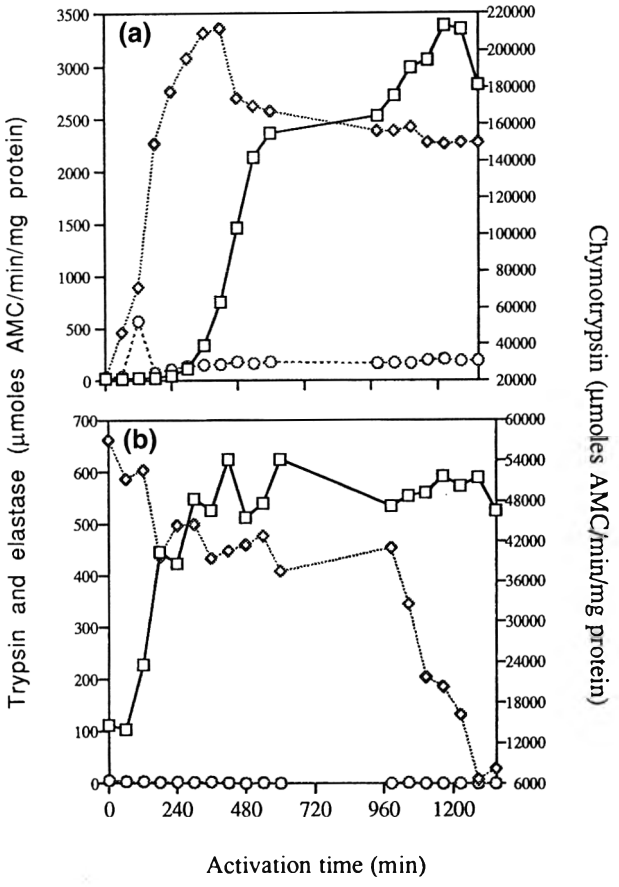

Fig. 1—Specific activity of trypsin —□—, chymotrypsin ·····◇····· and elastase ----○---- using fluorogenic substrates (μ moles AMC/min/mg protein) in the extraction fractions, Sample B (a) and the dilute form of 30S (b) over time at pH 7.0 and 37°C.

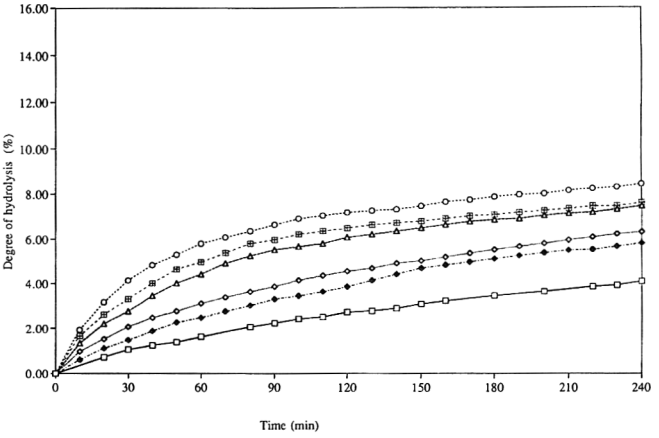

Fig. 2—Time course hydrolysis of lactalbumin by extraction fraction Sample B and 30S, at different activation levels: Sample B (4 hr) —□— ; 30S (4 hr) -·-·◆-·-· ; 30S (16 hr) ----○---- 30S (28 hr) ---□--- ; 30S (44 hr) ----△---- and 30Snc (4 hr) ·····◇····· , (activated in the absence of Ca ions). Reaction conditions: 8% (w/v) substrate conc; E:S (as in Table 2), pH 8.0, 50°C.

Activation of pancreatic proteinases was independent of exogenous trypsin addition. However, activation was slower if not initiated by trypsin, as shown previously (Desnuelle and Rovery, 1961). During activation, time-dependent changes occurred in the ratios of pancreatic endoproteinases in Sample B and 30S during 37°C incubation [Fig. 2 (a) and (b)]. Each proteinase activity reached a maximum at different times. In sample B, trypsin activity started to increase in a linear fashion after 300 min incubation, reaching maximal activity after 1220 min incubation. In the absence of exogenous trypsin, however, this was delayed by 125 min. In sample B, the ratio of trypsin to chymotrypsin and elastase after 1220 min incubation was in the order of 17:740:1 μmole AMC/min/mg protein. After that time, the trypsin activity began to decrease. Chymotrypsin activity reached a maximum after 240 min after which the activity started to decrease reaching 149,235 μmoles/min/mg protein at 1225 min. Elastase activity appeared to remain constant after 245 min. The activity of the endoproteinases differed greatly in the dilute solution of saturated ammonium sulfate, i.e., 30S [Fig. 2 (b)] from that in the crude sample B [Fig. 2 (a)]. Trypsin activity increased and reached a maximum at ~ 650 units after 600 min and remained stable close to this level over an extended period. Chymotrypsin activity was very unstable during 37°C activation of the 30S fraction. The activity decreased rapidly from the beginning of the process. After 600 min the endoproteinase activity ratios were as follows: trypsin: chymotrypsin: elastase (625:38,000:1). Minimal elastase activity was detected.

Chymotrypsin and elastase appearred to autolyze or deactivate at a faster rate than trypsin [Fig. 2 (b)]. The low levels of elastase activity [Fig. 2 (b)] could be attributed to dilution of stabilizing influences which may prevent autolysis. Ammonium sulfate and other salts are known to inactivate elastase activity (Lewis et al., 1959; Lamy et al., 1961; Gertler and Birk, 1970).

In our study, removal of ammonium sulfate by desalting had no effect on elastase activity (Table 1); however, de-activation of elastase activity may have occurred prior to desalting.

A concentrated, desalted 30S fraction was used for further study on the effects of activation time on endoproteolytic activities. By extension of activation time, the ratios of endoproteinases was changed (Table 3). Furthermore, the hydrolysis curves (Fig. 1) and the products (Table 4 and 5) from these proteolytic preparations used in the hydrolysis of lactalbumin were different for each activation time (Table 4).

Characterization of lactalbumin hydrolysates

Final percentage degrees of hydrolysis and molecular distribution. The hydrolysis curves obtained on incubation of lactalbumin with sample B (Fig. 1) were compared with those using concentrated desalted and activated 30S freeze-dried preparations (Fig. 3) and commercial proteases (Fig. 4) under different conditions. The molecular weight distribution data (Table 4) are averages for two elutions of a given hydrolysate. Peak areas for a specific molecular weight range were expressed as percentage of total peak area obtained at 280 nm.

The 30S extract had more activity on lactalbumin than sample B as seen from the higher degree of hydrolysis obtained after incubation for 240 min (Fig. 1 and Table 2). Sample B hydrolysate showed a low % DH after 240 min as compared to 30S. However, the E:S ratio for sample B used in hydrolysis was calculated on the basis of protein, whereas the other preparations were used on the basis of weight of powder. Sample B hydrolysates displayed large peak areas corresponding to peptides within the molecular weight ranges of 13,300–570 Da (28.3%). Less material was produced in the <570 Da region. In comparison to the cruder Sample B profile, the 30Snc activated for 4 hr showed a reduction in peak area in the 13,300–570 Da range and an increase in peak area corresponding to <570 Da region. The peptide distributions for 30S and 30nc were different. Considerably less peptides occurred in the 13,000–570 Da molecular weight region for 30Snc than for 30S.

We studied different activation times as a means of producing a specific ratio of endoproteinases. Hydrolysates produced using these preparations revealed significant changes in molecular characteristics, i.e., with respect to % DH (Fig. 1), molecular weight distribution of peptides (Table 4), and levels of free amino acids generated (Table 5). As activation time was increased the final % DH increased up to 16 hr (Table 2). At longer activation times, i.e., 28 and 44 hr, the final % DH was decreased. When the 30S activation times were increased, i.e.,

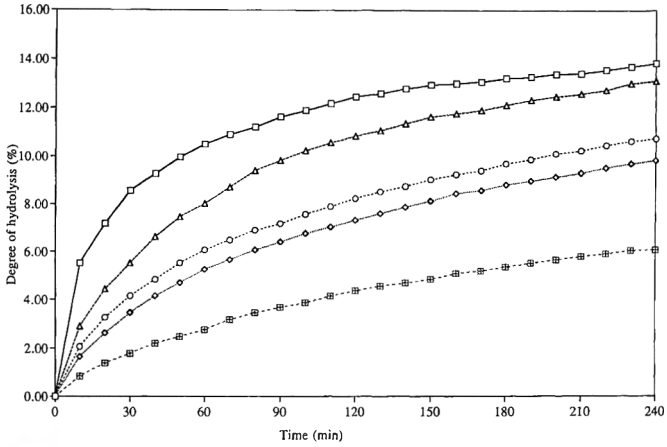

Fig. 3—Time course hydrolysis of lactalbumin by Corolase PP preparations pretreated at different times: 0hr —□—; 4hr ----△----; 16hr ----○---- ; 28hr ·····◇····· ; 44hr ---□--- (indicated in Table 2). Reaction conditions: 8% (w/v) substrate conc; E:S (as in Table 2), pH 8.0, 50°C.

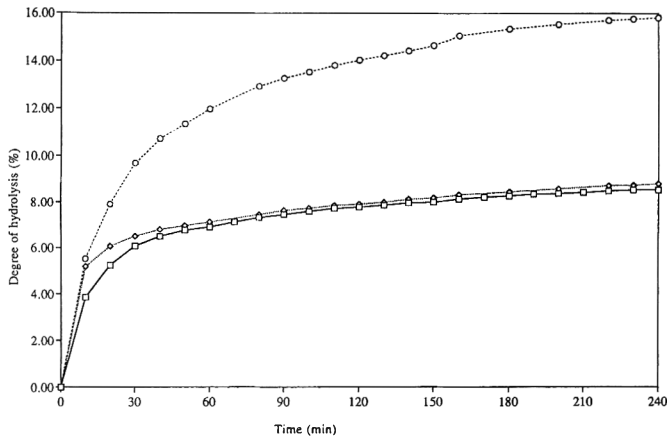

Fig. 4—Time course hydrolysis of lactalbumin by commercial preparations without pretreatment Corolase PP ---○--- ; PTN 3.0S ····◇···· ; PEM 25C0S —□— (indicated in Table 2), as a function of time. Reaction conditions: 8% (w/v) substrate conc; E:S (as in Table 2), pH 8.0, 50°C.

Table 3—Proteolytic activity (μmoles AMC/min/mg protein) after different activation times for the concentrated, desalted 30S preparation

Activation time (hr)	Trypsin	Chymotrypsin	Elastase
	μmoles AMC/min/mg protein		
0[a]	—	—	—
4	2.10	879.63	0.30
16	39.90	1008.23	0.41
28	52.80	1440.33	0.51
44	84.90	1152.26	0.31

[a] In the absence of activation, no endoproteolytic activity was detected.

Table 4—Molecular weight distribution (%)[1] of protein components in lactalbumin hydrolysates prepared using commercial pancreatic protease preparations and pancreatic extracts activated for different times, E:S ratio as given in Table 2

		Molecular weight range (Da)		
		29,200-13,300	13,300-570	< 570
Commercial prepn	Pretreatment time (hr)			
Corolase PP	0 hr	0.26 ± 0.07[2]	6.98 ± 0.67	92.90 ± 0.12
Corolase PP	4 hr	0.68 ± 0.29	17.75 ± 0.10	81.52 ± 0.09
Corolase PP	16 hr	0.94 ± 0.05	21.79 ± 0.36	77.10 ± 0.20
Corolase PP	28 hr	0.58 ± 0.36	24.88 ± 0.03	74.40 ± 0.09
Corolase PP	44 hr	0.38 ± 0.00	32.54 ± 0.14	67.10 ± 0.82
PTN 3.0S	0 hr	0.01 ± 0.01	25.00 ± 1.42	75.01 ± 0.20
PEM 2500S	0 hr	0.00 ± 0.00	23.46 ± 0.22	76.43 ± 0.22
Pancreatic extracts	Activation time (hr)			
Sample B	4 hr	0.01 ± 0.01	28.32 ± 0.53	76.13 ± 2.16
30S nc[3]	4 hr	0.01 ± 0.01	15.70 ± 0.01	83.92 ± 0.03
30S TPA	4 hr	0.04 ± 0.01	1.45 ± 0.12	98.12 ± 0.05
30S	4 hr	0.00 ± 0.00	26.95 ± 0.30	72.77 ± 0.11
30S	16 hr	0.00 ± 0.00	22.52 ± 0.16	77.18 ± 0.33
30S	28 hr	0.05 ± 0.05	20.21 ± 0.83	79.75 ± 0.22
30S	44 hr	0.00 ± 0.05	10.01 ± 0.09	89.99 ± 0.56

[a] Integrated peak areas between defined elution volumes corresponding to particular molecular weight distribution was calculated using Minichrom®.
[b] Mean value from 2 repetitions ± standard deviation.
[c] Proteolytic extract activated in the absence of calcium ions.

16, 28 and 44 hr, a corresponding increase in hydrolytic activity occurred (Table 3). Note the small reduction of peak area within the 13,300–570 Da range (Table 4). The high final % DH of 30S (16 hr) was apparently contradictory to other data (Table 4). The preparations 30S (28 hr) and 30S (44 hr), which had lower final % DH values, had higher hydrolytic activity in the 13,300–570 Da range. This may indicate the formation of different active forms of proteinases during activation of those preparations (Vestling et al., 1990).

Table 5—Free amino acids (mg/g protein) in lactalbumin hydrolysates prepared using pancreatic extracts activated for different times

Amino acid	Sample B 4	30S nc[b] 4	30S 4	30S 16	30S 28	30S 44
Asp	—[a]	—	—	—	—	—
Thr	—	—	—	—	—	—
Ser	—	—	—	—	0.15	—
Glu	—	—	—	—	—	—
Pro	—	—	—	—	—	—
Gly	—	—	—	—	—	—
Ala	0.36	0.36	0.34	0.35	0.37	0.32
Met	—	—	—	—	—	—
Val	—	—	—	—	0.10	—
Ile	—	—	—	—	—	—
Leu	—	—	—	0.13	0.14	0.08
Tyr	—	—	—	—	—	—
His	—	—	—	—	—	—
Lys	—	—	—	1.20	0.59	0.86
Trp	—	—	—	—	—	—
Arg	—	—	—	—	—	—

[a] not detected
[b] 30S nc = 30S preparation activated without calcium.

Table 6—Free amino acids (mg/g protein) in lactalbumin hydrolysates prepared using commercial preparations pretreated Corolase PP

Amino acid	PTN 3.0S	PEM 2500S	Corolase PP Pretreatment time (hr) 0	4	16	28	44
Asp	—[a]	—	0.47	0.43	0.37	0.33	0.23
Thr	—	—	1.08	1.01	0.58	0.49	0.29
Ser	—	—	1.86	2.21	1.18	1.04	0.63
Glu	—	—	—	0.02	0.12	0.10	—
Pro	—	—	—	—	—	—	—
Gly	—	—	—	—	0.22	0.26	—
Ala	0.29	0.29	1.44	1.12	0.84	0.85	0.70
Met	—	—	3.19	3.07	1.87	1.73	0.58
Val	—	—	9.12	7.85	4.56	4.01	3.26
Ileu	—	—	4.86	4.38	2.69	2.50	1.31
Leu	0.49	0.47	24.37	19.99	11.75	10.28	6.06
Tyr	—	—	15.91	14.26	10.24	9.11	5.75
Phe	—	—	12.04	10.83	8.73	7.91	5.94
His	—	—	2.06	1.66	1.17	0.99	1.00
Lys	6.36	4.30	36.84	32.37	21.42	17.44	10.07
Trp	—	—	—	—	—	—	—
Arg	0.12	3.16	13.79	12.33	9.47	6.19	6.19

[a] not detected.

Similarities occurred between 30S (16 hr), PEM 2500S and PTN 3.0S as seen from the molecular weight profiles of peptides and hydrolysis curves produced by those preparations (Table 4, Fig. 1 and 4, respectively). This may be due to the presence of two main endoproteolytic activities, i.e., trypsin and chymotrypsin, in 30S (16 hr) (Table 3), in PEM 2500S and PTN 3.0S

(Mullally et al., 1994). This was confirmed by the low concentrations of free amino acids in hydrolysates produced by those preparations (Table 5 and 6). PEM 2500S and PTN 3.0S appeared to have similar activity against lactalbumin (Fig. 4) on the basis of total proteolytic activity (Table 2). The curves for treatment of lactalbumin by PTN 3.0S and PEM 2500S began to plateau after 40–50 min, while the DH values after 240 min were 8.83 and 8.57, respectively. The 30S purification sample was presumably approaching the ratios of proteinases in commercial preparations when the zymogen activation time was increased to 16 hr, (as indicated seen by very similar final DH values). The final DH value of 30S sample activated for 16 hr was 8.44%. (Table 2).

Pretreatment of a solution (10 mg/mL) of commercial protease preparation (Corolase PP) by holding at 37°C, prior to hydrolysis of lactalbumin, showed that, as time of pretreatment increased, the degree of hydrolysis decreased (Table 2). Increasing holding times had a progressively negative effect on initial rates of hydrolysis and final DH values after 240 min incubation. Material corresponding to the 13,300–570 Da and <570 Da molecular weight region was hydrolyzed to a lesser extent with increasing holding times (Table 4). For 16, 28 and 44 hr samples a decrease occurred in areas corresponding to low-molecular-weight peptides as holding time was extended.

Production of free amino acids. The concentrations of amino acids in free solution following incubation of lactalbumin with pancreatic extracts and commercial preparations were compared (Tables 5 and 6). Concentrations <0.006 (mg/g) were not included. All preparations produced hydrolysates which contained alanine in free solution. Both sample B and 30S activated for 4 hr produced hydrolysates which only had alanine free in solution. There was no difference in the hydrolysate produced by the 30S preparation activated for 4 hr in the presence and absence of Ca ions (Table 5). All enzyme preparations, when applied to lactalbumin hydrolysis on the basis of total proteolytic activity, produced hydrolysates deficient in glutamic acid and glycine. All hydrolysates contained leucine, with exception of that produced by 30S, activated for 4 hr and 16 hr, 30S activated in the absence of Ca ions and Sample B. As time of activation of zymogen forms of 30S preparation was increased, several additional free amino acids were released into the hydrolysates, especially lysine and leucine. The hydrolysates produced by Corolase PP contained the following amino acids: lysine > leucine > tyrosine > arginine > phenylalanine > valine > isoleucine > methionine > serine > histidine > alanine > threonine. The concentration of each amino acid was reduced with increasing pretreatment time of the Corolase PP preparation, although the levels of each appeared to remain in proportion (Table 6). The analysis of free amino acids in the hydrolysates of Corolase PP and 30S preparations revealed significant differences. The low levels of free amino acids in hydrolysates produced by 30S indicated an absence of exopeptidase activity contrary to levels known to be present in Corolase PP (Mullally et al., 1994). Corolase PP also had elastase activity and low levels of this activity in 30S may have contributed to similarities between preparations with respect to shape of DH curves (Fig. 1 and 3).

Different published extraction procedures have been used for purification of individual pancreatic proteinases. Acidic extraction has been used in the initial step for trypsin and chymotrypsin recovery, whereas acetone powder extraction was used in protocols for elastase, carboxypeptidase and exopeptidase recovery (Northrop et al., 1948; Laskowski, 1956; Lewis et al., 1959; Folk and Schirmer, 1963). In our study proteolytic preparations Sample B and 30S were prepared using an initial acid extraction step. This may have destroyed exopeptidase activities and certain endoproteinase activities. Porcine chymotrypsin B and C are acid-labile (Folk, 1970). Incubation of elastase at very low pH values for extended times deactivated the enzyme (Lewis et al., 1956). This would, therefore, explain the absence of most of the free amino acids in the hydrolysates and the extremely low levels of amino acids, such as alanine. Levels of carboxypeptidases are higher in porcine tissues (Desnuelle and Rovery, 1961), although carboxypeptidase B is subject to de-activation at very low pH values and, therefore, the purification procedure we employed would not be suitable for its isolation. All these factors considered, it is unlikely that exopeptidase activity was prominent in the 30S preparation.

CONCLUSION

The ratio of proteinases in a pancreatic enzyme preparation was related not only to the degree of purification or source of pancreatic tissue but also to the activation procedure employed. Unless an extensive purification protocol involving chromatographic procedures is employed, it seems unlikely that a precise ratio of endoproteinases (trypsin, chymotrypsin and elastase) could be maintained. Zymogen activation alone, however, can control the ratio of proteinases present in an enzymatic preparation, and thus the physicochemical properties of any resultant whey protein hydrolysate. During hydrolysis of proteins, if a number of enzymatic activities work in conjunction with one another, the resulting hydrolysates differ. Properties of hydrolysates are dependent on several factors, such as the number and ratio of proteases, enzyme specificity, enzyme stability, and, possibly, auto-digestion. To find a desirable ratio of endoproteinases within a proteolytic preparation, a compromise between activation and de-activation must be found.

REFERENCES

Abita, J.P., Delaage, M., and Lazdunski, M. 1969. The mechanism of activation of trypsinogen. The role of the four N-terminal aspartyl residues. European J. Biochem. 8: 314–324.

Adler-Nissen, J. 1986. *Enzymic Hydrolysis of Food Proteins*. Elsevier Applied Science Publishers, London.

AOAC 1980. *Offical Methods of Analysis* (13th ed). Association of Official Analytical Chemists, Washington, DC.

Arai, S. and Fujimaki, M. 1991. Enzymatic modification of proteins with special reference to improving their functional properties. In *Food Enzymology-2*, P.F. Fox (Ed.), p. 83–104. Elsevier Applied Science Publishers, London.

Asselin, J., Amiot, J., Gauthier, S.F., Mourad, W., and Hebert, J. 1988. Immunogenicity and allergenicity of whey protein hyrolysates. J. Food Sci. 53: 1208–1211.

Asselin, J., Hébert, J., and Amiot, J. 1989. Effects in in vitro proteolysis on the allergenicity of major whey proteins. J. Food Sci. 54: 1037–1039.

Balls, A.K. 1965. Concerning trypsinogen. Proc. Natl. Acad. Sci. U.S. 53: 392–396.

Bergmann, M. and Fruton, J.S. 1941. The specificity of proteinases. Advan. Enzymol. 1: 63–98.

Buck, F.F., Vithayathil, A.J., Bier, M., and Nord, F.F. 1962. On mechanisms of enzyme action. LXXIII. Studies on trypsins from beef, sheep and pig pancreas. Arch. Biochem. Biophys. 97: 417–424.

Clemente, F., De Caro, A., and Figarella, C. 1972. Composition du suc pancréatique humain. Eur. J. Biochem. 31: 186–193.

Dagorn, J.C. and Lahaie, R.G. 1981. Dietary regulation of pancreatic protein sythesis. I Rapid and specific modulation of enzyme synthesis by changes in dietary composition. Biochim. Biophys. Acta 654: 111–118.

Desnuelle, P. and Rovery, M. 1961. The proteins of the exocrine pancreas. Adv. Protein. Chem. 16: 139–195.

Folk, J.E. 1970. Individual proteolytic enzymes—chymotrypsin C (porcine pancreas). In *Methods in Enzymology Vol XIX—Proteolytic Enzymes*, G.E. Perlmann and L. Lorand (Ed), p. 109–112. Academic Press, New York.

Folk, J.E. and Schirmer, E.W. 1963. The porcine pancreatic carboxypeptidase A system. I Three forms of the active enzyme. J. Biol. Chem. 238: 3884–3894.

Fox, P.F. 1991. *Food Enzymology*. Elsevier Applied Science Publishers, London.

Fullbrook, P., Pawlett, D., and Parker, D. 1987. The aminopeptidases: a novel group of enzymes with applications within the food industry. In *Novel Biotechniques and Processes for the Food Industry*, p. 79–85. On Line Publications, London.

Garcia de Fernando, G.D. and Fox, P.F. 1991. Extracellular proteinases from *Micrococcus GF*: Isolation and characterisation. Le Lait 71: 371–382.

Gertler, A. and Birk, Y. 1970. Isolation and characterisation of porcine proelastase. Eur. J. Biochem. 12: 170–176.

Gertler, A., Weiss, Y., and Burstein, Y. 1977. Purification and characterisation of porcine elastase II and investigation of its elastolytic specificity. Biochemistry 16: 2709–2716.

Godfrey, T. and Reichelt, J. 1983. *Industrial Enzymology*. The Nature Press, New York,

Grimble, G.K. and Silk, D.B.A. 1989. Milk protein and enteral and parenteral feeding in disease. In *Milk Proteins: Nutritional, Functional and Technological Aspects*, C.A. Barth and E. Schlimme (Ed.), p. 270–281. Steinkopff Verlag, Darmstadt, Germany.

Guash, A., Coll, M., Avilés, F.X., and Huber, R. 1992. Three-dimensional structure of porcine pancreatic procarboxypeptidase A. A comparison of the A and B zymogens and their determinants for inhibition and activation. J. Mol. Biol. 224: 141–157.

Hartley, B.S. and Shotton, D.M. 1971. Elastase. In *The Enzymes—Vol. III. Hydrolysis: Peptide Bonds*, P.D. Boyer (Ed.), p. 323–373. Academic Press, New York.
Hakim, A.A., Peters, R.L., Samsa, E.G., and van Melle, P.J. 1962. Heterogeneous natures of trypsins. Enzymologia 25: 134–154.
Jost, R., Meister, N., and Monti, J.C. 1988. Procédé de préparation d'un hydrolysat de protéines de lactosérum et d'un aliment hypoallergéniques. European Patent 0 322 589.
Jost, R., Monti, J.C., and Pahud, J.J. 1977. Partial enzymatic hydrolysis of whey protein by trypsin. J. Dairy Sc. 60: 1387–1393.
Keil, B. 1971. Trypsin In *The Enzymes—Vol. III Hydrolysis: Peptide Bonds*, P.D. Boyer (Ed.), p. 250–272. Academic Press, New York.
Kuehler, C.A. and Stine, C.M. 1974. Effect of enzymatic hydrolysis on some functional properties of whey protein. J. Food Sci. 39: 379–382.
Lakkis, J. and Villota, R. 1992. Comparative performance of chemically and enzymatically modified proteins. Food Chemistry 43: 93–105.
Lamy, F., Craig, C., and Tauber, S. 1961. Studies on elastase and elastin. I. Assay and properties of elastase. J. Biol. Chem. 236: 86–91.
Largmann, C., Brodrick, J.W., and Geokas, M.C. 1976. Purification and characterisation of two human pancreatic elastases. Biochemistry 15: 2491–2500.
Laskowski, M. 1956. Trypsin. In *Methods in Enzymology. Vol. II*, S.P. Colowick and N.O. Kaplan (Ed.), p. 9–25. Academic Press, New York.
Lazdunski, M. and Delaage, M. 1965. Sur la morphologie des trypsins de porc et de boeuf étude des dénaturations reversibles. Biochim. Biophys. Acta 105: 541–561.
Lewis, U.J., Williams, D.E., and Brink, N.G. 1959. Pancreatic elastase: Differentiation from other pancreatic proteases. J. Biol. Chem. 234: 2304–2307.
Marchis-Mouren, G. 1965. Protéines: Étude comparée de l'équipement enzymatique du suc pancréatique de diverses espèces. Bull. Soc. Chim. Biol. 47: 2207–2217.
Martínez, M.C., Avilés F.X., Sansegundo, B., and Cuchillo, C.M. 1981. Comparison between the monomeric and binary—complex forms of procarboxypeptidase A from whole pig pancreas. Biochem. J. 197: 141–147.
Maubois, J.L. and Léonil, J. 1989. Peptides du lait à activitié biologique. Le Lait 69: 245–269.
Merritt, R.J., Carter, M., Haight, M., and Eisenberg, L.D. 1990. Whey protein hydrolysate formula for infants with gastrointestinal intolerance to cow milk and soy protein in infant formulas. J. Pediat. Gastroent. Nutr. 11: 78–82.
Mullally, M.M., O'Callaghan, D.M., FitzGerald, R.J., Donnelly, W.J., and Dalton, J.P. 1994. Proteolytic and peptidolytic activities in commercial pancreatic protease preparation and their relationship to some whey protein hydrolysate characteristics. J. Agr. Food Chem. 42: 2973–2981.
Mulvihill, D.M. 1992. Milk protein products. In *Advances Dairy Chemistry-1*, P.F. Fox (Ed.), p. 369–404, Elsevier Applied Science, London.
Murray, T.K. and Baker, B.E. 1952. Studies on protein hydrolysis. 1. Preliminary observations on the taste of enzymic protein hydrolysates. J. Sci. Food Agric. 3: 470–475.
Northrop, J.H., Kunitz, M., and Herriott, R.M. 1948. Trypsin. In *Crystalline Enzymes*, 2nd ed. p. 16. Columbia Univ. Press, New York.
Padfield, P.J. and Case, R.M. 1987. Release of individual enzymes from guinea pig pancreas following stimulation with CCK-8 or CCK-33. Pancreas. 2: 439–446.
Peanasky, R.J., Gratecos, D., Baratti, J., and Rovery, M. 1969. Mode of activation and N-terminal sequence of subunit II in bovine procarboxypeptidase A and of porcine chymotrypsinogen C. Biochim. Biophys. Acta 181: 82–92.
Plainer, H. and Sprossler, B. 1990. Debittering of protein hydrolysates by a new technical exopeptidase preparation. Food Biotechnol. 4: 366.
Poulsen, O. 1987. A process for the preparation of a heat resistant non-bitter water soluble peptide product, the product produced by the process, and nutrients, refreshments and dietetics comprising the product. European Patent WO 87/03785.
Puigserver, A., Chapus, C., and Kerfelec, B. 1986. Pancreatic exopeptidases. In *Molecular and Cellular Basis of Digestion*, P. Desnuelle, H. Sjostrom, H., and O. Noren (Ed.), p. 235–247. Elsevier Science Publishers, London.
Robinson, B.P., Short, J.L., Marshall, K.R. 1976. Traditional lactalbumin manufacture, properties and uses. N.Z.J. Dairy Technol. 11: 114–126.
Schroeder, D.D. and Shaw, E. 1968. Chromatography of trypsin and its derivatives. Characterisation of a new active form of bovine trypsin. J. Biol. Chem. 243: 2943–2949.
Smith, R.L. and Shaw, E. 1969. Pseudotrypsin. A modified bovine trypsin produced by limited autodigestion J. Biol. Chem. 244: 4704–4712.
Thibault, P.A. 1991. Partial hydrolysate of whey proteins, enzymatic process for the preparation of this hydrolysate, and hypoallergenic dietetic milk containing it. US Patent, 4,981,704.
Turgeon, S.L., Gauthier, S.F., and Paquin, P. 1992. Emulsifying property of whey peptide fractions as a function of pH and ionic strength. J. Food Sci. 57: 601–604.
Uriel, J. and Avrameas, S. 1965. Systematic fractionation of swine pancreatic hydrolases. I Fractionation of enzymes soluble in ammonium sulfate solution at 0.40 saturation. Biochemistry 4: 1740–1749.
Vegarud, G.E. and Langsrud, T. 1989. The level of bitterness and solubility of hydrolysates produced by controlled hydrolysis of caseins. J. Dairy Res. 56: 375–379.
Vestling, M.M., Murphy, C.M., and Fenselau, C. 1990. Recognition of trypsin autolysis products by high-performance liquid chromatography and mass spectrometry. Anal. Chem. 62: 2391–2394.
Vithayathil, A.J., Buck, F., Bier, M., and Nord, F.F. 1961. On the mechanism of enzyme action. LXXII Comparative studies on trypsins of various origins. Arch. Bioch. Biophys. 92: 532–540.
Walsh, K.A. 1970. Trypsinogens and trypsins of various species In *Methods in Enzymology. Vol. XIX—Proteolytic Enzymes: The Serine Proteases*, G.E. Perlmann and L. Lorand (Ed.). p. 41–63. Academic Press, New York.
Wilcox, P.E. 1970. Chymotrypsinogens—Chymotrypsins. In *Methods in Enzymology. Vol. XIX—Proteolytic Enzymes: The Serine Proteases*, G.E. Perlmann and L. Lorand (Ed.) p. 64–112. Academic Press, New York.
Zimmerman, M., Ashe, B., Yurewicz, E.C., and Patel, G. 1977. Sensitive assays for trypsin, elastase and chymotrypsin using fluorogenic substrates. Anal. Biochem. 73: 47–51.
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Antioxidative Activity of Maillard Reaction Products in Cooked Ground Beef, Sensory and TBA Values

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ABSTRACT

Maillard reaction products (MRPs) obtained by autoclaving egg albumin acid hydrolysate and glucose for 1 hr were added to ground beef which was cooked and stored at 4°C for 8 days. The antioxidative activity of MRPs was investigated by a trained sensory panel and the TBA test. The panel evaluated samples for loss of desirable cooked beef notes and generation of off-flavors, such as cardboard and painty. With added 1% MRPs, painty aroma and flavor scores were low and did not ($p > 0.05$) change over time. The coefficients of determination were high ($r^2 = 0.92$ to 0.77, $p < 0.0001$) between aroma and flavor scores for any two similar attributes. The TBA values decreased by 17% with the addition of 0.5% MRP and by 39% with 1% MRP over time.

Key Words: ground beef, Maillard products, antioxidants, sensory quality

INTRODUCTION

CONSUMPTION of cooked ground beef has increased because of ease of preparation, versatility, and good value. However, this product is susceptible to warmed-over flavor (WOF), also referred to as meat flavor deterioration (MFD), and oxidative rancidity. MFD develops in cooked meats during short periods of refrigerated storage (Asghar et al., 1988; St. Angelo et al., 1992). The most likely cause of MFD development is oxidation of membrane phospholipids (Igene and Pearson 1979).

As lipids oxidize, a mixture of aldehydes and ketones is produced. These classes of compounds are responsible for development of undesirable flavor attributes, such as cardboard and painty. Their concentrations increase, and desirable flavors, such as cooked beef, decrease during storage (St. Angelo et al., 1992; 1990). MFD appears to result from this combined chemical process in which lipid oxidation products are increased and desirable meaty flavor compounds are diminished (St. Angelo et al., 1992).

Use of antioxidants is a means of preventing or decreasing oxidative rancidity in cooked ground beef during storage. Synthetic antioxidants, such as butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT), effectively prevent lipid oxidation in cooked meat products (Chastain et al., 1982; Chen et al., 1984). Testing antioxidants from natural sources as alternatives to prevent MFD in cooked meat products may be advantageous because of increasing sensitivity of consumers to synthetic additives. Thus, the application of natural antioxidants for prevention of MFD has received considerable attention (Al-Jalay et al., 1987; Stoick et al., 1991).

Maillard reaction products (MRPs) comprise one group of antioxidants that may be considered natural because the reactions occur normally in foods. Several reports have discussed the use of MRPs formed from amino acids and sugars as antioxidants in model systems (Lingnert and Eriksson, 1980a, b; Beckel and Waller, 1983). The addition of MRPs prepared from histidine and glucose or enzymatic hemoglobin hydrolysate and glucose has improved the oxidative stability of sausage during frozen storage (Lingnert and Lundgren, 1980). Bailey et al. (1987) found that MRPs prepared from histidine and glucose were effective inhibitors of oxidative rancidity in cooked ground beef.

Our objective was to evaluate the effects of MRPs prepared from egg albumin hydrolysate and glucose on development of oxidative rancidity of cooked ground beef stored at 4°C, as measured by sensory aroma and flavor and by the thiobarbituric acid (TBA) test. Egg albumin was chosen as the source of precursor amines because it is readily available and MRPs from it can be readily produced. In addition previous work (Alfawaz et al., 1994) has shown that MRPs from egg hydrolysate were very potent antioxidants as measured by TBA values and volatiles.

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MATERIALS & METHODS

Preparation of protein hydrolysate

Protein hydrolysates were prepared similar to procedures described by Alfawaz et al. (1994). Egg albumin (80% protein, Henningsen Foods Inc, Omaha, NE) was suspended in 1.0N hydrochloric acid (Fisher Scientific, Fair Lawn, NJ) and refluxed for 7 hr. Extent of hydrolysis was monitored using the trinitrobenzenesulfonic acid (TNBS) method (Adler-Nissen, 1979).

Preparation of Maillard reaction products (MRPs)

MRPs were obtained by autoclaving 25 mL of distilled water containing 2g glucose (Holly-Harneds of Wichita, Wichita, KS) and 2g egg albumin hydrolysate (1.92 amino equiv/g protein) at 121°C and ≈1kg/cm² for 1 hr. Initial pH of the reaction mixture was adjusted to 9.0 with 1N NaOH. The MRPs were lyophilized at −40°C for 36 hr and then flushed with nitrogen prior to storage at −13°C. One gram of MRPs was diluted (20 times) with distilled water and the browning intensity of the MRPs was measured at 420 nm with a Perkin-Elmer Spectrophotometer (Model 552, Coleman Instruments Division, Oak Brook. IL).

Preparation of cooked ground beef

Four beef top rounds (Kansas State University, Meat Laboratory) were obtained from four carcasses, and each was chosen randomly to represent a replicate. Each top round was trimmed of all visible fat and connective tissue and ground through a 0.4 cm plate with a food processor (Model 2250, Rival Manufacturing Co, Kansas City, MO). The ground round (1.5–2.2% fat) was divided randomly into 300-g portions for each storage time, vacuum packaged, and stored at −13°C for no longer than 8 days. The frozen meat was thawed at 4°C for 24 hr. then MRPs at 1 or 0.5% were dissolved in distilled water (10 mL of distilled water/100g ground beef) and added to each of the treatments. Control samples contained 10% distilled water but no MRPs. The ground beef was placed in a 600 mL beaker and cooked in a water bath at 80°C to a final internal temperature of 70°C. The cooked ground beef was stored at 4°C for 4 or 8 days and evaluated by sensory, TBA, and color analysis. Freshly cooked ground beef prepared as described above immediately before analysis served as day 0 samples.

Sensory evaluation

Orientation. Sensory evaluations were conducted using a five-member trained panel (Kansas State University, Sensory Analysis Center). The panelists were trained previously and had experience in evaluation of oxidative rancidity in cooked meat products. The panelists were oriented during one 3-hr session using fresh and rancid cooked ground beef as well as reference standards to enable them to develop and correctly use the descriptive terms. A wet brown grocery bag in a covered glass

Table 1—Effect of MRPs prepared from egg albumin hydrolysate and glucose on aroma scores[d] of cooked ground beef stored at 4°C

Sensory attributes	Treatment	Time (days) 0	4	8
Cooked lean beef	Control	8.85^{ax}	2.88^{ay}	2.08^{ay}
	0.5% MRPs	8.46^{ax}	3.93^{aby}	3.40^{aby}
	1% MRPs	8.47^{ax}	4.75^{by}	4.20^{by}
Browned/roasted	Control	0.28^{ax}	0.80^{ax}	0.45^{ax}
	0.5% MRPs	4.32^{bx}	2.23^{by}	1.98^{by}
	1% MRPs	5.90^{cx}	4.25^{cy}	2.45^{bz}
Cardboard	Control	0.00^{ax}	4.20^{ay}	2.28^{bz}
	0.5% MRPs	0.00^{ax}	3.38^{ay}	4.05^{ay}
	1% MRPs	0.00^{ax}	2.40^{by}	4.10^{az}
Painty	Control	0.00^{ax}	4.65^{ay}	6.28^{az}
	0.5% MRPs	0.00^{ax}	1.68^{by}	2.85^{by}
	1% MRPs	0.00^{ax}	0.33^{bx}	1.63^{bx}

[a-c] Means within the same column of the same attribute, with different letter are significantly different (P<0.05).
[d] Evaluations expressed on a 15-point numerical scale, where 0 = none and 15 = extreme.
[x-z] Means within the same row of the same attribute, with different letter are significantly different (P<0.05).

Table 2—Effect of MRPs from egg albumin hydrolysate and glucose on flavor scores[d] of cooked ground beef stored at 4°C

Sensory attributes	Treatment	Time (days) 0	4	8
Cooked lean beef	Control	8.85^{ax}	4.05^{by}	3.13^{by}
	0.5% MRPs	8.55^{ax}	5.83^{ay}	5.20^{ay}
	1% MRPs	8.43^{ax}	6.23^{ay}	6.08^{ay}
Browned/roasted	Control	0.40^{bx}	1.55^{bx}	0.73^{bx}
	0.5% MRPs	5.31^{ax}	4.93^{ax}	3.83^{abx}
	1% MRPs	7.48^{ax}	7.20^{ax}	4.95^{ax}
Cardboard	Control	0.00^{ax}	3.88^{ay}	2.70^{ay}
	0.5% MRPs	0.00^{ax}	2.48^{aby}	3.53^{ay}
	1% MRPs	0.00^{ax}	1.63^{bxy}	2.60^{ay}
Painty	Control	0.00^{ax}	2.70^{ay}	5.05^{az}
	0.5% MRPs	0.00^{ax}	1.48^{ay}	1.83^{by}
	1% MRPs	0.00^{ax}	0.33^{bx}	1.15^{bx}
Sour	Control	1.65^{ax}	1.13^{ay}	1.08^{ay}
	0.5% MRPs	1.05^{bx}	0.90^{ax}	1.08^{ax}
	1% MRPs	0.85^{bx}	0.93^{ax}	1.03^{ax}
Bitter	Control	0.00^{ax}	1.03^{ay}	1.68^{az}
	0.5% MRPs	0.05^{ax}	0.55^{by}	0.50^{by}
	1% MRPs	0.13^{ax}	0.18^{bx}	0.38^{bx}

[a-c] Means within the same column of the same attribute, with different letter are significantly different (P<0.05).
[d] Evaluations expressed on a 15-point numerical scale, where 0 = none and 15 = extreme.
[x-z] Means within the same row of the same attribute, with different letter are significantly different (P<0.05).

cup was the reference for cardboard aroma, and latex paint was the reference for painty aroma. Dilute acetic acid (0.03–0.08%) was the reference for sour flavor, and dilute caffeine (0.03–0.08%) was used for bitter flavor. Cooked lean beef attribute was used to describe the highly desirable, freshly cooked, beef flavor. The flavors associated with addition of MRPs were described by panelists in terms of browned/roasted. Cardboard and painty terms were used to describe oxidative rancidity notes (Johnsen and Civille, 1986).

Sample preparation. About 40-g portions of cooked ground beef were placed in 3-ounce plastic cups and covered with 10-cm watch glasses. Samples were identified by 3-digit random numbers and presented in random order. The cooked meat was reheated in a microwave oven at high power for 15 sec immediately before serving to panelists.

Sample presentation. The cooked ground beef was evaluated using a 15-point, numerical, descriptive, analysis scale with end-anchors of 0 = none and 15 = extreme. The panelists first were asked to sniff the cooked ground beef and report their response for aroma attributes. For flavor attributes, panelists tasted the cooked ground beef. Sensory analysis was conducted in a room equipped with fluorescent light, with the panelists sitting at a round table. The sensory-panel sessions were conducted at 9:00 a.m. for four consecutive days, and samples were served 6 min apart. Freshly cooked ground beef was presented as the reference for fresh cooked beef aroma and flavor three times during each taste session.

Thiobarbituric acid (TBA) values

TBA values were determined on cooked ground beef by the distillation method of Tarladgis et al. (1960). Briefly, 10g of cooked ground beef were weighed into a 250 mL Kemmerer-Hallet distillation flask of a microKjeldahl steam distillation unit (Fisher Scientific, Fair Lawn, NJ). To the distillation flask, 46 mL distilled water, 3 mL of 4N hydrochloric acid, and a few drops of 1% antifoam A (Sigma Chemical, St. Louis, MO) were added. Fifty mL of distillate was collected in 8 min. To 5 mL of distillate, 5 mL of 0.02 M TBA reagent, prepared in distilled water, was added The solution was mixed and placed into a boiling water bath for 35 min and then cooled in an ice bath. Absorbance was read at 530 nm or. the Perkin-Elmer Spectrophotometer, and concentration of malonaldehyde was calculated from a standard curve obtained using solutions of 1,1,3,3-tetraethoxypropane (Sigma Chemical. St. Louis, MO). The TBA values were expressed as mg malonaldehyde/kg meat. Each sample was assayed in triplicate.

Table 3—Coefficients of determination between mean sensory aroma and flavor scores of cooked ground beef stored at 4°C

Aroma attributes	Flavor attributes: Cooked lean beef	Browned/roasted	Cardboard	Painty
Cooked lean beef	0.92	—	—	—
Browned/roasted	—	0.89	—	—
Cardboard	—	—	0.77	—
Painty	—	—	—	0.85

[a] All coefficients of determination are significant at p<0.0001, n = 180.

Table 4—Effect of MRPs prepared from egg albumin hydrolysate and glucose on TBA values (mg malonaldehyde/kg meat) of cooked ground beef stored at 4°C

Treatment	Time (days) 0	4	8	% Inhibition[d]
Control	0.29^{ax}	5.19^{ay}	6.72^{az}	
0.5% MRPs	0.25^{ax}	3.98^{by}	5.60^{bz}	17.00
1% MRPs	0.23^{ax}	2.95^{cy}	4.10^{cz}	39.00

[a-c] Means within the same column of the same attribute, with different letter are significantly different (P<0.05).
[d] % inhibition = 1 − (TBA value of treatment/TBA value of control) × 100.
[x-z] Means within the same row of the same attribute, with different letter are significantly different (P<0.05).

Statistical analysis

The study was designed as a randomized complete block with four replicates. Each replicate was treated as a block and treatments (0, 0.5, and 1% MRPs) and storage time (0, 4, and 8 days) as main effects. Sensory data and TBA values were analyzed by analysis of variance using the General Linear Model (GLM) Procedure of the Statistical Analysis System (SAS Institute, Inc., 1989). Interaction between treatment level and storage time was tested. To account for variation among sensory panelists, they were included in the model. Comparisons between treatments for each day were evaluated by the Least Square Means Procedure (SAS Institute, Inc., 1989). Differences between days for each treatment also were tested using the Least Square Means Procedure. Differences were considered significant when means of compared sets differed at $P < 0.05$. Pearson coefficients of determination were computed for aroma and flavor scores and for TBA values and aroma and flavor scores.

RESULTS & DISCUSSIONS

Effect of MRPs on aroma profiles

The antioxidative activities of 0.5 or 1% MRPs prepared from egg albumin hydrolysate and glucose effects on aroma scores of cooked ground beef during storage at 4°C were compared (Table 1). As oxidative rancidity developed in control samples, cooked lean beef aroma decreased by 3.1-fold, while cardboard aroma increased by 4.25-fold and painty aroma by 4.65-fold at day 4. At day 8, the cooked lean beef aroma continued to decrease ($p > 0.05$), cardboard aroma began to decrease ($p < 0.05$), and painty aroma continued to increase ($p < 0.05$). These data agreed very well with results of St. Angelo et al. (1990), which showed that scores for cooked beef broth flavor decreased, cardboard flavor increased then leveled off, and painty flavor continued to increase during 5 days storage at 4°C.

Table 5—Coefficients of determination between TBA values (mg malonaldehyde/kg meat) and mean sensory aroma and flavor of cooked ground beef stored at 4°C[a]

Sensory attributes	TBA values
Aroma profile	
Cooked lean beef	−0.93
Cardboard	0.79
Painty	0.83
Flavor profiles	
Cooked lean beef	−0.94
Cardboard	0.79
Painty	0.70
Sour	−0.16
Bitter	0.79

[a] All coefficients of determination are significant at $p<0.0001$, $n = 36$.

The two treatments processed with 0.5 and 1% MRPs showed a decrease ($p < 0.05$) in scores for cooked lean beef aroma over 4 days, but this was not significant at day 8. The intensity of cooked lean beef aroma was significantly higher ($p < 0.05$) in samples processed with 1% MRPs than in the control samples for all storage times, except at day 0 (Table 1). The reduction in cooked lean beef aroma for samples containing 1% MRPs was not significant ($p > 0.05$) as compared to that for samples with 0.5% MRPs. The cooked ground beef processed with MRPs, which can function as free radical scavengers (Namiki, 1988); electron donators (Sato et al., 1973; Eichner, 1981); or chelators (Johnson et al., 1983), had low scores for cardboard and painty aroma. Comparison of antioxidative activity of MRPs at 0.5 and 1% showed no significant differences for reduction in cooked lean beef aroma, formation of painty aroma, or formation of cardboard aroma throughout storage except for formation of cardboard aroma at day 4.

Effect of MRPs on flavor profiles

The purpose of conducting the aroma, as well as flavor studies, was to determine if trained panelists could detect development of oxidative rancidity by smelling meat products without tasting them. The antioxidative effects of 0.5 or 1% MRPs from the egg albumin hydrolysate and glucose, on flavor scores of cooked ground beef during storage at 4°C were compared (Table 2). At day 0, control and samples treated with MRPs had scores of 8.85 to 8.43 for cooked lean beef flavor and scores of 0.0 for both cardboard and painty flavors. This trend confirmed aroma scores (Table 1). The control, which did not contain MRPs, lost 50% of the original cooked lean beef flavor within 4 days. In the control, the cardboard and painty flavor scores increased rapidly ($p < 0.05$) throughout storage. The pattern of change for flavor scores was identical to that for aroma scores. The addition of MRPs at concentrations of 0.5 and 1% significantly slowed the rate of diminishing cooked lean beef flavor compared to the control at days 4 and 8.

The intensity of painty flavor was higher than that of cardboard flavor in the control samples at day 8. However, in treated samples, the intensity of cardboard flavor was higher than that of painty flavor (Table 2). These results may indicate that the cardboard flavor was stabilized at higher concentrations in the treated samples than in controls. This trend agreed with data from aroma scores (Table 1). These data were in agreement with earlier reports by St. Angelo et al. (1990) that the intensity scores of cardboard flavor in cooked beef patties treated with rosemary were higher than those of painty flavor.

Scores for browned/roasted flavor and aroma, which were associated with the addition of MRPs, were higher ($p < 0.05$) in treated samples than in the control (Table 1 and 2). The browned/roasted scores decreased ($p < 0.05$) for samples treated with 1 or 0.5% MRPs over storage. The browned/roasted scores in the control sample were low and did not change ($p < 0.05$) throughout storage.

Sour and bitter taste scores were also compared (Table 2). The sour score in the control decreased ($P < 0.05$) during storage, but with no significant change from day 4 to 8. The sour scores in samples treated with 1 or 0.5% MRPs were low and did not change ($P < 0.05$) throughout storage. The bitter scores in the control increased ($P < 0.05$) during storage. Sour and bitter scores have been previously shown to be lower for cooked ground beef treated with antioxidants and vacuum packaged (St. Angelo et al., 1992).

Bailey et al. (1987) found that the addition of MRPs prepared from histidine and glucose was effective in retarding oxidative rancidity in cooked ground beef stored at 4°C. They demonstrated that MRPs caused about a 10 fold reduction in oxidation products, hexanal and 2,3-octanedione. The reduction of oxidation products correlated well with sensory data, however, only the general attribute, warmed over flavor, was evaluated. Sato et al. (1973) demonstrated that the addition of MRPs formed from glycine or lysine and glucose to cooked ground beef inhibited oxidative rancidity during 2 days storage at 4°C. Lingnert and Lundgren (1980) found that preformed MRPs from histidine and glucose or enzymatic hemoglobin hydrolysate and glucose were effective antioxidants in sausage during frozen storage, as determined by sensory evaluation.

Relationship between aroma and flavor scores

A second objective of our study was to correlate aroma and flavor sensory attributes. The relationship between any two similar aroma and flavor attributes was significant ($p < 0.0001$) in cooked ground beef stored at 4°C (Table 3). The coefficients of determination (r^2) between two similar attributes for aroma and flavor were very strong and ranged from 0.92 to 0.77. These results suggested that, with a highly trained panel, smelling meat products may be useful for studying oxidative rancidity as well as or instead of tasting them.

TBA values

Mean TBA values for freshly cooked ground beef were between 0.29 and 0.23, with no significant differences ($p > 0.05$) among treatments (Table 4). TBA values increased over 8 days storage for all treatments, with controls oxidized most rapidly ($p < 0.05$) and to the greatest extent between days 0 and 4. When 1% MRPs were added to the cooked ground beef, TBA values were 43% lower for day-4 samples and 39% lower for those stored for 8 days, compared to controls.

Relationship between TBA values and sensory scores

A third objective was to correlate TBA values and sensory scores. Significant coefficients of determination ($P < 0.0001$) were found between sensory scores and TBA values (Table 5). The sensory scores for desirable cooked lean beef negatively correlated ($r^2 = -0.93$) to TBA values. The oxidative rancidity descriptive terms (cardboard, painty, and bitter) positively correlated ($r^2 = 0.70$ to 0.83) to TBA values. These results agreed very well with St. Angelo et al. (1987, 1992) in that most undesirable flavor notes in oxidized cooked ground beef correlated highly with chemical markers, including TBA values. Note that St. Angelo et al. (1992) also found a similar, weaker correlation of flavor notes cardboard, bitter and sour to markers such as hexanal and TBA numbers. Ang and Lyon (1990) reported high correlation coefficients in cooked poultry between sensory scores of flavor notes cardboard, rancid, and painty and TBA values and headspace volatiles.

CONCLUSIONS

MRPs FROM EGG ALBUMIN acid hydrolysate and glucose have potent antioxidative activity, which can retard development of oxidative rancidity in cooked ground beef during storage at 4°C. Sensory aroma and flavor scores for any two similar attributes correlated highly. This suggests that aroma evaluation of espe-

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Radiation Sensitivity of *Listeria monocytogenes* on Beef as Affected by Temperature

D.W. THAYER and G. BOYD

ABSTRACT

Longissimus dorsi from beef was inoculated with *Listeria monocytogenes* and the effect of gamma irradiation on the survival of this pathogen at −60 to +15°C was determined. Radiation D-values were determined for inactivation of *L. monocytogenes* at 5°C intervals from −20 to 5°C. These data were used to develop an equation to predict the response to gamma radiation within that range. An abrupt increase in resistance occurred at −5°C. The radiation D-value was 0.45 kGy at 0°C, 0.77 kGy at −5°C and 1.21 kGy at −20°C. A straight line was obtained when the $\log_{10}$ of the D-values from −5 to −20°C was plotted vs the reciprocal of the absolute temperature. This led us to calculate a value analogous to the Arrhenius activation energy for inactivation of *L. monocytogenes* by gamma radiation.

Key Words: beef, irradiation, food safety, *Listeria*, meat

INTRODUCTION

LISTERIA MONOCYTOGENES has caused periodic outbreaks of foodborne disease with considerable mortality and is of particular concern in ready-to-eat refrigerated foods because it can multiply at refrigeration temperatures (Broome et al., 1990; Palumbo, 1986). This concern caused the Food & Drug Administration and the Food Safety & Inspection Service of the U.S. Department of Agriculture to establish zero tolerances for *L. monocytogenes* in ready-to-eat foods. Most outbreaks of listeriosis were related to products such as cole slaw, milk, and cheese; however, undercooked wieners, sausages, poultry, and shellfish also have been associated with outbreaks of the disease (Cantoni et al., 1989; Centers for Disease Control, 1989; Pearson and Marth, 1990; Schwartz et al., 1988). *L. monocytogenes* have been found on raw meat and poultry and on their products such as paté, turkey franks, fermented sausages, and cooked beef (Bailey et al., 1989; Carosella, 1990; Farber et al., 1989; Genigeorgis et al., 1989; Roberts, 1994; Wenger et al., 1990).

Gamma or electron-beam irradiation treatment of meat was proposed as a method for elimination of *L. monocytogenes* (Beuchat et al., 1993; Grant and Patterson, 1992; Huhtanen et al., 1989; Patterson, 1989; Patterson et al., 1993). With exception of Beuchat et al. (1993) no other workers considered the effects irradiation temperature might have on survival of the organism. Because of possible effects temperature may have introduced (from 2–4°C to 12°C, Huhtanen et al., 1989; Patterson, 1989; Patterson et al., 1993), it was not valid to compare results from these studies. Beuchat et al. (1993) reported on the survival of *L. monocytogenes* on ground beef at irradiation temperatures from −17 to −15°C and from 3 to 5°C but did not find a significant temperature effect. However, significant effects of irradiation temperature were found on the survival of *Campylobacter jejuni* (Clavero et al., 1994), *Clostridium botulinum* spores (El-Bisi et al., 1966), *Escherichia coli* O157:H7 (Clavero et al., 1994; Thayer and Boyd, 1993; Stapleton and Edington, 1953), *Salmonella* (Thayer and Boyd 1991a,b; Thayer et al., 1990), and *Staphylococcus aureus* (Thayer and Boyd, 1992). Because temperature-dependent radiation sensitivity for both gram-positive and negative bacteria has been demonstrated, we initiated a study of the effects of irradiation temperature on survival of *L. monocytogenes*. Our objective was to test the "null hypothesis" that the resistance of *L. monocytogenes* to gamma radiation was not affected by the temperature of the sample during irradiation.

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MATERIALS & METHODS

Cultures

Listeria monocytogenes 15313, 43256, 49594, and 7644 were obtained from the American Type Culture Collection, Rockville, MD. All cultures were maintained and cloned on Tryptic Soy Agar (TSA, Difco, Detroit, MI). Culture identity was confirmed by gram stains and from reactions on GNI or GPI cards of the Vitek AMS Automicrobic System (bioMérieux Vitek, Inc., USA, Hazelwood, MO) (Aldridge et al., 1977; Knight et al., 1990), as appropriate. Each member of the pathogen mixture was propagated independently in 100 mL of tryptic soy broth (TSB, Difco, Detroit, MI) at 35°C with agitation at 150 rpm on a rotary shaker for 18 hr at 35°C. Equal amounts from each culture were mixed, and cultures were harvested by centrifugation. A ten-fold inoculum was prepared by resuspending the cells in 1/10 volume of Butterfield's phosphate (0.25M KH_2PO_4 adjusted to pH 7.2 with NaOH).

Substrates and packaging conditions

Beef (steer) was obtained the day after slaughter from Carl Venezia of Conshohocken, PA. The Longissimus dorsi was carefully trimmed of fat, cubed, and frozen in dry ice. The meat was then pulverized while frozen in a Hobart silent cutter to yield a homogeneous material. The meat was subdivided into 100 ± 0.05g amounts, spread thinly, and vacuum sealed in Stomacher 400 polyethylene bags (Tekmar Co., Cincinnati, OH). The bags were then vacuum sealed in high barrier pouches fabricated with 0.025 mm polycaprolactam (nylon 6) as the outside layer, 0.0090 mm aluminum foil as the middle layer, and 0.051 mm polyethylene terephthalate as the inner layer (American National Can Company, Des Moines, IA) to provide better protection during handling and to prevent oxygen transmission to the samples. The meat was frozen at −50°C and sterilized by gamma irradiation to a dose of 42 kGy at −30°C. Prior research (Thayer et al., 1987, 1990; Thayer and Boyd, 1991a,b) demonstrated that such treatments did not significantly alter the wholesomeness and nutritional characteristics or the response of *Salmonella typhimurium* on chicken meat to gamma radiation. Both the sterile and nonsterile meat were stored at −50°C prior to sterilization and use.

Radiation source and irradiation techniques

The self-contained gamma-radiation source of ^{137}Cs had a strength of ~134,000 Ci (4.95 PBq) and a dose rate of 0.108 kGy min^{-1}. The dose rate was established using National Physical Laboratory (Middlesex, U.K.) dosimeters. Variations in absorbed doses given to experimental samples were minimized by placement within a uniform portion of the radiation field. Samples were maintained at the selected temperature ±0.5°C during irradiation by injecting the gas phase from liquid nitrogen into the irradiation chamber. Temperature was monitored continuously during irradiation with a calibrated thermocouple placed directly on the sample.

Inoculation of meat for determination of D_{10} values

Sterile meat was inoculated with enough cells for a final population of ~10^9 stationary-phase cells/g (10 mL per 100g meat) and mixed in a sterile Number 400 polyethylene Stomacher bag for 90 sec using a Stomacher 400 (Tekmar Co., Cincinnati, OH). Aliquots of 5.0 ± 0.05g of

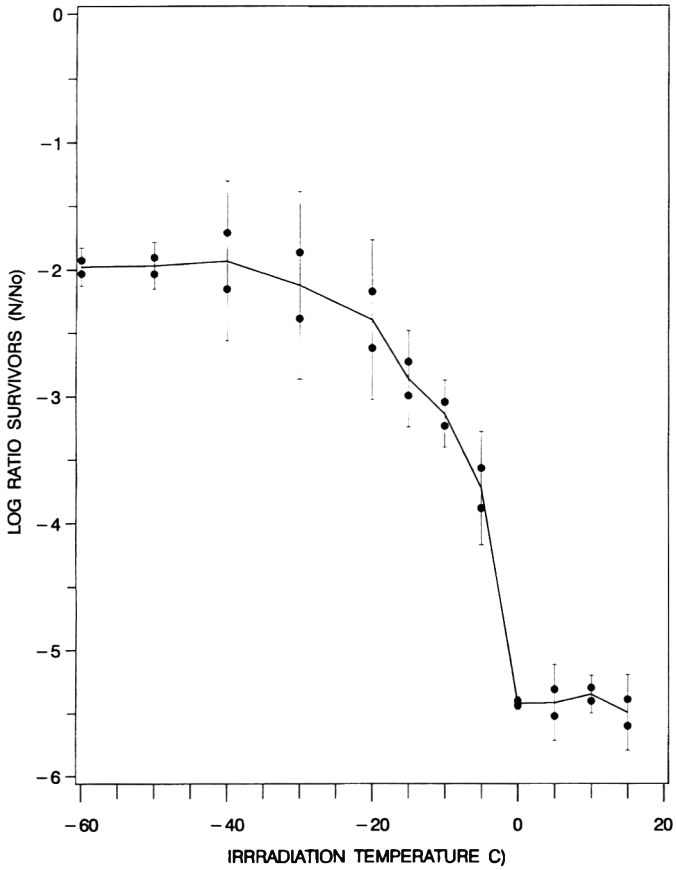

Fig. 1—Survival of *L. monocytogenes* on ground beef following gamma radiation dose of 2.0 kGy as related to temperature during irradiation.

inoculated meat were transferred aseptically to radiation-sterilized oxygen-permeable poultry bags (E-300, Cryovac Division, W.R. Grace & CO., Duncan, SC). The bags complied with U.S. regulations. Inoculated meat was spread uniformly over an area of about 10 × 10 cm within the bags and heat sealed *in vacuo*.

Effect of irradiation temperature on survival

Inoculated meat samples received a dose of 2.0 kGy at irradiation temperatures of 15, 10, 5, 0, −5, −10, −15, −20, −30, −40, −50, and −60°C. Two nonirradiated controls were prepared from the same inoculated meat; one was frozen and one was not. Controls were used to determine the number (N_0) of *L. monocytogenes* colony forming units (CFU) prior to treatment with gamma radiation for samples irradiated while nonfrozen or frozen. The frozen control tested for any effect due to freezing on viability of cells. The study was repeated twice.

Effect of irradiation temperature on D_{10} value

Inoculated meat samples received radiation doses of 0 to 3.0 kGy in increments of 0.60 kGy at 5, 0, −5, −10, −15, and −20°C. All samples for each replicate study were inoculated from the same inoculum. The studies were repeated twice.

Microbiological analysis

Samples were assayed for CFU by standard pour-plate procedures using TSA with serial dilutions in sterile Butterfield's phosphate. Petri plates containing *L. monocytogenes* were incubated for 48 hr at 37°C before counting. CFU were counted on three petri plates having 30–300 colonies with a New Brunswick Scientific Biotran II automated colony counter.

Statistical analysis

Cultural responses were expressed as the logarithm of CFU/g. For each experiment, the average (N) CFU for the three plate counts obtained for each replicate sample was determined and divided by the average of the three zero-dose values (N_o) to give a survivor value (N/N_o). The $\log_{10}$ survivor values ($\log_{10}(N/N_o)$) were then used for subsequent calculations. The D-values (dose in kGy resulting in 90% reduction of viable CFU at specific temperatures) were the reciprocals of the slopes of linear regressions of the log survivor values vs radiation dose determined by least squares analyses. Zero-dose values were excluded from the calculation of regression to avoid shoulder effects as described by Thayer et al. (1990). Regression techniques were used to fit second-order response-surface models (Draper and Smith, 1981). Statistical calculations were performed with the general linear models procedure of the SAS statistical package (Freund et al., 1986; SAS Institute, Inc., 1987). Regressions were tested for differences by analysis of covariance.

RESULTS

Effect of irradiation temperature on survival

Results of an irradiation dose of 2.0 kGy at irradiation temperatures from −60°C to +15°C were compared (Fig. 1). Almost no change occurred in the resistance of *L. monocytogenes* to gamma radiation between 15 and 0°C; however, between 0 and −5°C a very high increase in resistance occurred. Further increases in radiation resistance occurred between −5 and −40°C.

Effect of irradiation temperature on radiation D-value

Analysis of survival data at various doses, for the 2 replicate studies produced the following equation from which survival of *L. monocytogenes* at any temperature between 5 to −20°C and radiation doses of 0 to 3.0 kGy could be predicted:

$$Log_{10}\ survivors\ (N/N_0) = -0.1001 - 0.0650 \times temperature - 1.922 \times kGy - 0.0638 \times temperature \times kGy - 0.0036 \times temperature^2 \quad (1)$$

R^2 for this equation is 0.9470. With it we could make predictions as indicated (Fig. 2). Using Eq. (1) the logarithm of the predicted survival (N/N_o) of *L. monocytogenes* when irradiated to an absorbed dose of 2.4 kGy on beef at temperatures of −20, −15, −10, −5, 0, and 5°C is −1.791, −2.251, −2.892, −3.712, −4.713, and −5.894, respectively.

The D-value for *L. monocytogenes* did not change between irradiation temperatures of 5 and 0°C but increased almost threefold between 0 and −20°C (Table 1). When the $\log_{10}$ of radiation D-values for both replicate studies at each temperature were plotted vs the reciprocal of absolute temperature (K°) from −5 to −20°C they fell in a straight line (Fig. 3), from which the following regression equation with $R^2 = 0.953$ could be calculated where T = K°:

$$\log_{10} D\text{-}value = -3.539 + 917.3 \times (T^{-1}) \quad (2)$$

Eq. (2) could be used to calculate D-values at intermediate temperatures; but, more importantly, the slope could be converted to a value analogous to the Arrhenius activation energy (E_a):

$$k = se^{\frac{-E_a}{RT}} \quad (3)$$

Where s is a constant, E_a is the Arrhenius activation energy, R is the gas constant, and T is the absolute temperature (K°). If D-value is substituted for the rate constant and Eq. (3) is expressed in logarithmic form,

$$\log D\text{-}value = \frac{E_a}{2.303R} \times \frac{1}{T} + \log s \quad (4)$$

Differentiation of Eq. (4) with respect to temperature and integration yields,

$$\log \frac{D\text{-}value_2}{D\text{-}value_1} = \frac{E_a}{2.303R} \times \frac{T_2 - T_1}{T_1 T_2} \quad (5)$$

Using Eq. (5) the slope of the line (Fig. 3) is 917.3, and E_a has

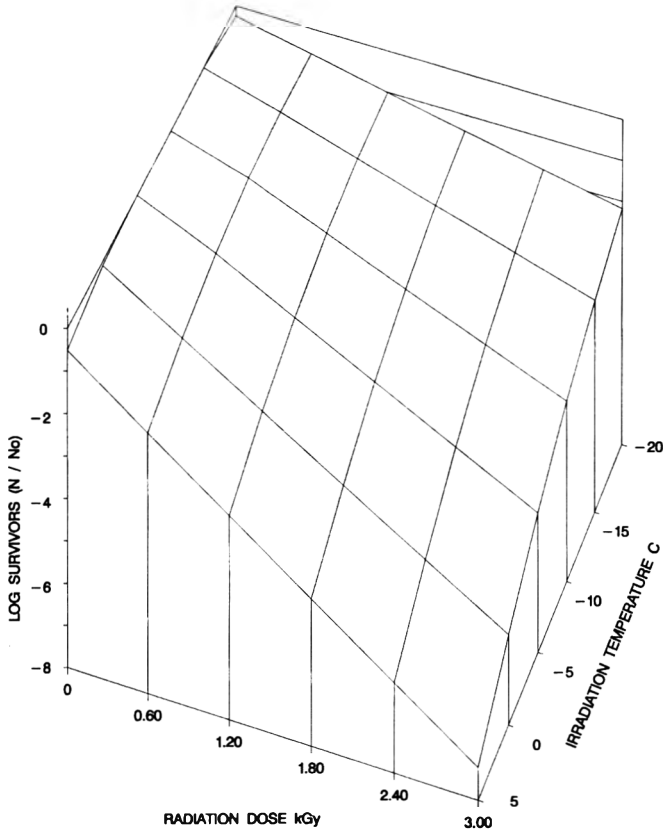

Fig. 2—Predicted survival of *L. monocytogenes* on ground beef following gamma radiation doses of 0 to 3.0 kGy at irradiation temperatures from −20 to +5°C.

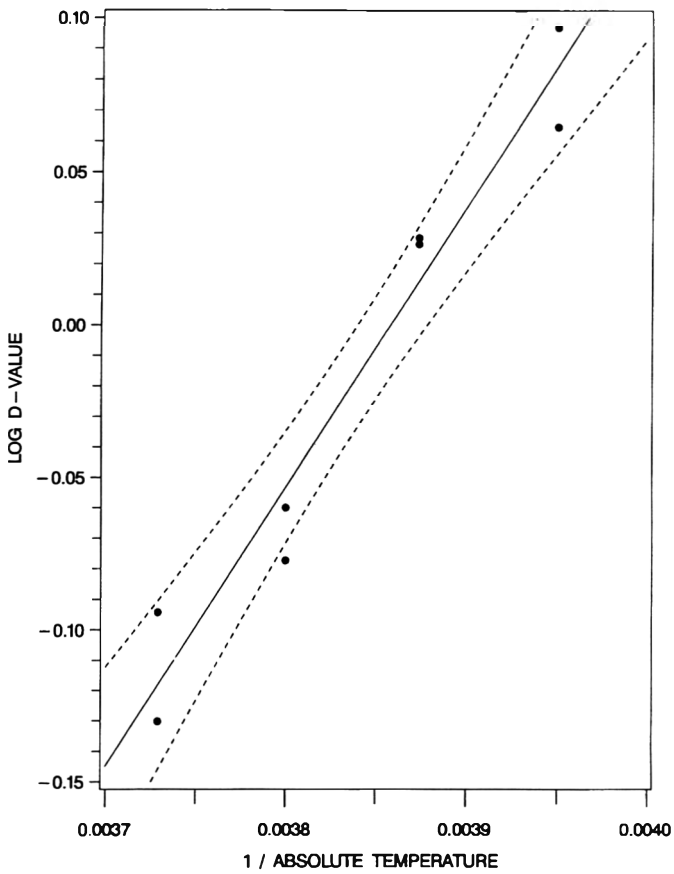

Fig. 3—Log D-values for *L. monocytogenes* on beef treated with gamma radiation as related to 1/T (absolute temperature).

Table 1—Gamma radiation resistance of *L. monocytogenes*[a] at different temperatures

Irradiation temp °C	1/T[b]	D-value ± SE[b] kGy	log D-value
+5	0.00360	0.445 ± 0.012	−0.352
0	0.00366	0.453 ± 0.016	−0.344
−5	0.00373	0.772 ± 0.046	−0.112
−10	0.00380	0.854 ± 0.046	−0.068
−15	0.00387	1.006 ± 0.036	0.028
−20	0.00395	1.208 ± 0.058	0.082

[a] *L. monocytogenes* ATCC 7644, 15313, 43256, and 49594 harvested at 16 hr and mixed with beef *Longissimus dorsi*.
[b] T = absolute temperature; SE = standard error.

the value of −2.303R (slope) = 17,564 joules deg^{-1} $mole^{-1}$. Since the D-values are expressed in kGy per log cell inactivation, and 1 Gy is equivalent to 1 joule/kg, E_a can also be expressed as 17.6 kGy g^{-1} deg^{-1} $mole^{-1}$.

DISCUSSION

RESULTS CLEARLY INDICATE a significant response of *L. monocytogenes* on beef to the temperature of irradiation; $10^{2.9}$ more cells would survive a dose of 2.4 kGy at −20°C than at 0°C. Billen (1987) estimated that ~85% of the potential damage to irradiated *E. coli* was due to radiolysis products of water, primarily OH·. The change in radiation sensitivity at subfreezing temperatures has been attributed to decreased OH·mobility (Taub et al., 1979). Beuchat et al. (1993) did not find a significant difference in radiation resistance of *L. monocytogenes* to gamma radiation on nonfrozen (2–5°C) and frozen (−17 to −14°C) hamburger. We postulate that the discrepancy between our results and those from Beuchat et al. (1993) may be due to recovery and possibly growth of injured *Listeria* in nonfrozen samples during the 12 to 14 hr storage and shipping time before samples (in the Beuchat et al., 1993 study) were analyzed. In our study, samples were analyzed immediately following irradiation, whereas in their study samples had been shipped from Florida to Georgia for analysis.

We obtained a D-value of 0.44 at +5°C, similar to values reported by Huhtanen et al. (1989) at 2–4°C on chicken of 0.46 kGy and Grant and Patterson (1992) at 3–4°C of 0.40 kGy. Equation (1) predicted that the effects of a radiation dose of 2.0 kGy at 3°C would be a reduction in number of surviving CFU by 4.55 log. The mean of the nonirradiated population of Huhtanen et al. (1989) was 8.50, which was reduced to 4.41 by irradiation to 2.0 kGy, a reduction of 4.19 log. The difference between the predicted reduction of viable CFU and the reported value were not great and could result from several factors, such as presence of oxygen during irradiation.

The linear increase in the $\log_{10}$ D-values from −5 to −20°C further supported the hypothesis that inactivation of bacteria even at temperatures below freezing are directly related to reaction temperature as occurs for chemical reactions. This also supports the concept that most cellular inactivation processes are due to interactions with radiolytic products of water rather than direct interaction of gamma radiation with DNA, since those reactions would not be temperature dependent. Our developed Eq. (1) could predict the response of *L. monocytogenes* on beef to gamma irradiation between the temperatures of 5 to −20°C. This equation indicates that products contaminated with *L. monocytogenes* and irradiated to a dose of 2.4 kGy even at −20°C would be much safer than nonirradiated products. However, much greater security could be provided by irradiation at temperatures of 0 to 5°C.

REFERENCES

Aldridge, C., Jones, P.W., Gibson, S.F., Lanham, J.W., Meyer, M.C., Vannest, R., and Charles, R.A. 1977. Automated microbiological detection/identification system. J. Clin. Microbiol. 6: 406.

Bailey, J.S., Fletcher, D.L., and Cox, N.A. 1989. Recovery and serotype distribution of *Listeria monocytogenes* from broiler chickens in the Southeastern United States. J. Food Prot. 52: 148.
Beuchat, L.R., Doyle, M.P., and Brackett, R.E. 1993. Irradiation inactivation of bacterial pathogens in ground beef. AMI Foundation, Arlington, VA.
Billen, D. 1987. Free radical scavenging and the expression of potentially lethal damage in X-irradiated repair-deficient *Escherichia coli*. Radiat. Res. 111:354.
Broome, C.V., Gellin, B., and Schwartz, B. 1990. Epidemiology of listeriosis in the United States. Ch. 10, in *Foodborne Listeriosis*, A.J. Miller, J.L. Smith, and G.A. Somkuti (Ed.), p. 61–65. Elsevier, Amsterdam, The Netherlands.
Cantoni, C., Balzaretti, C., and Valenti, M. 1989. A case of listeriosis following consumption of sausages. Arch. Vet. Ital. 40: 141.
Carosella, J.M. 1990. Occurrence of *Listeria monocytogenes* in meat and poultry. Ch. 24, in *Foodborne Listeriosis*, A.J. Miller, J.L. Smith, and G.A. Somkuti (Ed.), p. 165–173, Elsevier, Amsterdam, The Netherlands.
Centers for Disease Control. 1989. Listeriosis associated with consumption of turkey franks. Morbid. Mortal. Weekly Rep. 38: 267.
Clavero, M.R.S., Monk, J.D., Beuchat, L.R., Doyle, M.P., and Brackett, R.E. 1994. Inactivation of *Escherichia coli* O157:H7, Salmonellae, and *Campylobacter jejuni* in raw ground beef by gamma irradiation. Appl. Environ. Microbiol. 60: 2069.
Draper, N.R. and Smith, H. 1981. *Applied Regression Analysis*, 2nd ed. John Wiley & Sons, Inc., New York.
El-Bisi, H.M., Snyder, O.P., and Levin, R.E. 1966. Radiation death kinetics of *C. botulinum* spores at cryogenic temperatures. In *Botulism 1966, Proc. 5th Intl. Symp. Food Microbiol.*, M. Ingram and T.A. Roberts (Ed.), p. 89. Chapman and Hall, Ltd., London.
Farber, J.M., Sanders, G.W., and Johnston, M.A. 1989. A survey of various foods for the presence of *Listeria* species. J. Food Prot. 52: 456.
Freund, R.J., Littel, R.C., and Spector, P.C. 1986. *SAS System for Linear Models*. SAS Institute Inc., Cary, NC.
Genigeorgis, C.A., Dutulescu, D., and Garayzabal, J.F. 1989. Prevalence of *Listeria* spp. in poultry meat at the supermarket and slaughterhouse level. J. Food Prot. 52: 618.
Grant, I.R. and Patterson, M.F. 1992. Sensitivity of foodborne pathogens to irradiation in the components of a chilled ready meal. Food Microbiol. 9: 95.
Huhtanen, C.N., Jenkins, R.K., and Thayer, D.W. 1989. Gamma radiation sensitivity of *Listeria monocytogenes*. J. Food Prot. 52: 610.
Knight, M.T., Wood, D.W., Black, J.F., Gosney, G., Rigney, R.D., Agin, J.R., Gravens, C.K., and Frarnham, S.M. 1990. Gram-negative identification of *Salmonella, Escherichia coli*, and other *Enterobacteriaceae* isolated from foods: collaborative study. J. Assoc. Off. Anal. Chem. 73: 729.
Palumbo, S.A. 1986. Is refrigeration enough to restrain foodborne pathogens? J. Food Prot. 49: 1003.
Patterson, M. 1989. Sensitivity of *Listeria monocytogenes* to irradiation on poultry meat and in phosphate-buffered saline. Lett. Appl. Microbiol. 7: 55.
Patterson, M.F., Damoglou, A.P., and Buick, R.K. 1993. Effects of irradiation dose and storage temperature on the growth of *Listeria monocytogenes* on poultry meat. Food Microbiol. 10: 197.
Pearson, L.J. and Marth, E.H. 1990. *Listeria monocytogenes*—threat to a safe food supply: a review. J. Dairy Sci. 73: 912.
Roberts, D. 1994. *Listeria monocytogenes* and food: The U.K. approach. Dairy, Food, Environ. Sanitation. 14: 198.
SAS Institute Inc. 1987. *SAS/STAT® Guide for Personal Computers, Version 6 Edition*, p. 1028. SAS Institute Inc., Cary, NC.
Schwartz, B., Ciesielski, C.A., Broome, C.V., Gaventa, S., Brown, G.R., Gellin, B.G., Hightower, A.W., Mascola, L., and the Listeriosis Study Group. 1988. Association of sporadic listeriosis with consumption of uncooked hot dogs and undercooked chicken. Lancet ii: 779.
Stapleton, G.E. and Edington, C.W. 1953. Temperature dependence of bacterial inactivation by X-rays. J. Radiation Res. 1: 229.
Taub, I.A., Halliday, J.W., and Sevilla, M.D. 1979. Chemical reactions in proteins irradiated at subfreezing temperatures. Adv. Chem. Ser. 180: 109.
Thayer, D.W., Boyd, G., Muller, W.S., Lipson, C.A., Hayne, W.C., and Baer, S.H. 1990. Radiation resistance of *Salmonella*. J. Indust. Microbiol. 5: 383.
Thayer, D.W. and Boyd, G. 1991a. Effect of ionizing radiation dose, temperature, and atmosphere on the survival of *Salmonella typhimurium* in sterile, mechanically deboned chicken meat. Poult. Sci. 70: 381.
Thayer, D.W. and Boyd, G. 1991b. Survival of *Salmonella typhimurium* ATCC 14028 on the surface of chicken legs or in mechanically deboned chicken meat gamma irradiated in air or vacuum at temperatures of −20 to +20 C. Poult. Sci. 70: 1026.
Thayer, D.W. and Boyd, G. 1992. Gamma ray processing to destroy *Staphylococcus aureus* in mechanically deboned chicken meat. J. Food Sci. 57: 848.
Thayer, D.W. and Boyd, G. 1993. Elimination of *Escherichia coli* O157 in meats by gamma irradiation. Appl. Environ. Microbiol. 59: 1030.
Thayer, D.W., Christopher, J.P., Campbell, L.A., Ronning, D.C., Dahlgren, R.R., Thomson, G.M., and Wierbicki, E. 1987. Toxicology studies of irradiation-sterilized chicken. J. Food Prot. 50: 278.
Wenger, J.D., Swaminathan, B., Hayes, P.S., Green, S.S., Pratt, M., Pinner, R.W. Schuchat, A., and Boome, C.V. 1990. *Listeria monocytogenes* contamination of turkey franks: Evaluation of a production facility. 53: 1015.
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cially the attributes painty and cooked lean beef by a highly trained panel could be a useful method to study development of oxidative rancidity in cooked meat products and to evaluate potential antioxidants.

REFERENCES

Alder-Nissen, J. 1979. Determination of the degree of hydrolysis of food protein hydrolysates by trinitrobenzenesulfonic acid. J. Agric. Food Chem. 27: 1256–1262.
Alfawaz, M., Smith, J.S., and Jeon, I.K. 1994. Maillard reaction products as antioxidants in precooked ground beef. Food Chem. 51: 311–318.
Al-Jalay, B., Blank, G., McConnell, B., and Al-Khayat, M. 1987. Antioxidant activity of selected spices used in fermented meat sausage. J. Food Prot. 50: 25–27.
Ang, C.Y.W. and Lyon, B.G. 1990. Evaluation of warmed-over flavor during chill storage of cooked broiler, thigh and skin by chemical, instrumental and sensory methods. J. Food Sci. 55: 644–648.
Asghar, A., Gray, J.I., Buckley, D.J., Pearson, A.M., and Booren, A.M. 1988. Perspectives on warmed-over flavor. Food Technol. 42(6): 102–108.
Bailey, M.E., Shin-Lee, S.Y., Dupuy, H.P., St. Angelo, A.J., and Vercellotti, J.R. 1987. Inhibition of warmed-over flavor by Maillard reaction products. In *Warmed-Over Flavor of Meat*, A.J. St. Angelo and M.E. Bailey (Ed.), p. 237–266. Academic Press, Inc., Orlando, FL.
Beckel, R.W. and Waller, G.R. 1983. Antioxidative arginine-xylose Maillard reaction products: Conditions for synthesis. J. Food Sci. 48: 996–997.
Chastain, M.F., Huffman, D.L., Hsieh, W.H., and Cordary, J.C. 1982. Antioxidants in restructured beef/pork steaks. J. Food Sci. 47: 1779–1782.
Chen, C.C., Pearson, A.M., Gray, J.I., and Merkel, R.A. 1984. Effects of salt and some antioxidants upon the TBA numbers of meat. Food Chem. 14: 167–172.
Eichner, K. 1981. Antioxidative effect of Maillard reaction intermediates. Prog. Food Nutr. Sci. 5: 441–451.
Igene, J.O. and Pearson, A.M., 1979. Role of phospholipids and triglycerides in warmed-over flavor development in meat model systems. J. Food Sci. 44: 1285–1290.
Johnson, P.B. and Civille, G.V. 1986. A standardized lexicon of meat WOF descriptors. J. Sensory Stud. 1: 99–104.
Johnson, P.E., Lykken, G., Mahalko, J., Milne, D., Inman, L., Garcia, W.J., and Inglett, G.E. 1983. The effect of browned and unbrowned corn products on absorption of zinc, iron and copper in humans. In *The Maillard Reaction in Foods and Nutrition*, G.R. Waller and M.S. Feather (Ed.), p. 3349–3360. American Chemical Society, Washington, DC.
Lingnert, H. and Eriksson, C.E. 1980a. Antioxidative Maillard reaction products. I. Products from sugars and free amino acids. J. Food Proc. Preserv. 4: 161–172.
Lingnert, H. and Eriksson, C.E. 1980b. Antioxidative Maillard reaction products. II. Products from sugars and peptides or protein hydrolysates. J. Food Proc. Preserv. 4: 173–181.
Lingnert, H. and Lundgren, B. 1980. Antioxidative Maillard reaction products. IV. Application in sausage. J. Food Proc. Preserv. 4: 235–47.
Namiki, M. 1988. Chemistry of Maillard reactions: Recent studies on the browning reaction mechanism and the development of antioxidants and mutagens. Adv. Food Res. 32: 115–184.
SAS Institute Inc. 1989. *SAS® User's Guide: Statistics., Version 6.07*. SAS Institute, Inc., Cary, NC.
Sato, K., Hegarty, G.R., and Herring, H.K. 1973. The inhibition of warmed-over flavor in cooked meats. J. Food Sci. 38: 398–403.
St. Angelo, A.J., Vercellotti, J.R., Legendre, M.G., Vinnett, C.H., Kuan, J.W., James, C. Jr., and Dupuy, H.P. 1987. Chemical and instrumental analyses of warmed-over flavor in beef. J. Food Sci. 52: 1163–1168.
St. Angelo, A.J., Crippen, K.L., Dupuy, H.P., and James, C. 1990. Chemical and sensory studies on antioxidant-treated beef. J. Food Sci. 55: 1501–1505.
St. Angelo, A.J., Spanier, A.M., and Bett, K.L. 1992. Chemical and sensory evaluation of flavor in untreated and antioxidant-treated meat. In *Lipid Oxidation in Food*, A.J. St. Angelo (Ed.), p. 140–160. American Chemical Society, Washington, D.C.
Stoick, S.M., Gray, J.F., Brooren, A.M., and Buckley, D.J. 1991. Oxidative stability of restructured beef steaks processed with oleoresin rosemary and sodium tripolyphosphate. J. Food Sci. 56: 597–600.
Tarladgis, B.G., Watts, B.M. Younthan, M.T. and Dugan, L.R. 1960. A distillation method for the quantitative determination of malonaldehyde in rancid foods. J. Am. Oil Chem. Soc. 37: 44–48.
Yamaguchi, N., Koyama, Y., and Fujimaki, M. 1981. Fractionation and antioxidative activity of browning reaction products between D-xylose and glycine. In *Maillard Reactions in Food*, C. Eriksson (Ed.), p. 429–439. Pergamon Press, Oxford.

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Phytic Acid Protective Effect Against Beef Round Muscle Lipid Peroxidation

BEOM JUN LEE and DELOY G. HENDRICKS

ABSTRACT

Phytic acid, constituting 1–5% of many plant seeds and cereals, can form iron-chelates and inhibit lipid peroxidation. We measured thiobarbituric acid reactive substances (TBARS), an indication of lipid peroxidation, in beef homogenates to investigate the effects of phytic acid with various homogenizing buffers and at different pH levels. Phytic acid effectively inhibited either iron-induced or non-iron-induced lipid peroxidations. The effect of phytic acid was dose- and pH-dependent. Such inhibition of lipid peroxidation may help prevent deterioration of food quality.

Key Words: beef, round muscle, peroxidation, phytic acid

INTRODUCTION

MANY CLASSES OF LIPID PEROXIDATION PRODUCTS exert toxic effects in both whole animals and cellular systems (Halliwell and Gutteridge, 1986; Kubow, 1992; Pearson et al., 1983). Lipid peroxidation is a major cause of quality deterioration in stored foods, especially in muscle tissues (Harel et al., 1988; Pearson et al., 1983). Such deterioration includes changes in flavor, color, texture and nutritive value, or the production of toxic compounds (Wilson et al., 1976). Lipid peroxidation in meats has become an important problem in restructured and precooked food products (Akamittath et al., 1990; Pearson et al., 1983).

Oxidation of muscle lipids involves the peroxidation of polyunsaturated fatty acids, located in the membranes of muscle foods (Keller and Kinsella, 1973; Khayat and Schwall, 1983). Transition metals such as Fe^{2+} and Cu^{2+} as well as heme compounds are important in lipid peroxidation in skeletal muscle (Decker and Welch, 1990; Kanner et al., 1988a). However, the function of heme proteins or nonheme iron in lipid peroxidation in muscle tissues is uncertain (Love and Pearson, 1974). Some researchers indicate hemoproteins are prooxidants of muscle tissues (Fisher and Deng, 1977; Lee et al., 1975; Love, 1983; Wallace et al., 1982), while others indicate nonheme iron compounds are more important factors (Harel et al., 1988; Kwoh, 1971). Several processes, such as refrigeration and cooking, may increase degradation of heme compounds increasing free and lower-molecular-weight iron compounds which are hypothesized to be responsible for lipid peroxidation (Decker and Hultin, 1990; Kanner et al., 1988b; Schricker and Miller, 1983).

Phytic acid is a natural plant inositol hexaphosphate constituting 1–5% of most cereals, nuts, legumes, oil seeds, pollen, and spores. Phytic acid can tightly bind to metal ions, and the metal phytate complexes are highly insoluble over a wide pH range (Graf and Eaton, 1990). Therefore, phytate has been hypothesized to interfere with mineral bioavailability in humans and animals (Ellis et al., 1982). Phytic acid is a powerful inhibitor of iron-driven hydroxyl radical formation because it can form a unique iron chelate that becomes catalytically inactive (Graf et al., 1987). Phytic acid may decrease the incidence of human colon cancer (Graf and Eaton, 1985; Shamsuddin et al., 1988) and fibrosarcoma (Jariwalla et al., 1988) in rats, perhaps due to the reduction of hydroxyl radical formation and decreased cell division.

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Our objective was to investigate the effects of phytic acid on lipid peroxidation in beef round muscle homogenate. In stored foods, mixed diets, and the gastrointestinal tract, phytic acid may be important in protecting against lipid peroxidation and against toxic effects of products formed by lipid peroxidation.

MATERIALS & METHODS

Materials

Phytic acid, 2-thiobarbituric acid, ferrozine [3-(2-pyridyl)-5,6-bis(4-phenylsulfonic acid)-1,2,4-triazine], tetraethoxypropane (TEP), butylated hydroxytoluene (BHT), Tris (hydroxymethyl) aminomethane, Hepes (N-2-hydroxyethyl piperazine N'-2-2-ethanesulfonic acid), and Pipes [piperazine-N,N'-bis(2-ethanesulfonic acid)] were purchased from Sigma Chemical Company (St. Louis, MO). Trichloroacetic acid (TCA) and sodium hydroxide were obtained from EM Science (Cherry Hill, NJ). Hydrogen peroxide, sodium phosphate (dibasic anhydrous), nitric acid, ammonium acetate, ascorbic acid, and ethylenediaminetetraacetic acid disodium salt (EDTA) were obtained from Mallinckrodt Inc. (Paris, KY).

Methods

Sample preparation. Beef round muscle (select grade) was purchased from Smith's Food and Drug Co., Logan, UT. Fat was removed, and the muscle was chopped into small pieces using a stainless steel knife. The chopped muscle was homogenized in deionized water (DW) for iron assay or in selected buffer solutions (50 mM) with a polytron homogenizer (Omni 5000 International Co., Waterburg, CT) for 1 min at level 2. The pH of the homogenate was adjusted with 0.5N HCl or 0.1N NaOH using a pH meter (Orion Research Incorporated Co., Boston, MA). The incubation mixture included 0.8 mL of 20% (w/v) beef homogenate and 0.2 mL of DW or 0.2 mL of phytic acid solution and was incubated for 60 min at 37°C. After incubation the homogenate was used for the thiobarbituric acid (TBA) test. All data were derived from triplicate samples.

Chemical analysis. TBARS assay was performed as described by Buege and Aust (1978). The reaction mixture contained 1.0 mL of incubation mixture and 2.0 mL of TCA-TBA-HCl stock solution. The mixture was heated for 20 min in a boiling water bath (95°C) to develop a pink color, cooled with tap water, centrifuged at 5,500 rpm for 15 min, and measured spectrophotometrically at 532 nm with a UV 2100 U spectrophotometer (Shimadzu Co., Kyoto, Japan). TBARS were calculated from a standard curve of malondialdehyde, a breakdown product of TEP. Total iron concentration was determined in wet-ashed samples by using ferrozine (Stookey, 1970). Each homogenized sample (0.5 mL) was digested with 3 mL of concentrated nitric acid and 0.2 mL of 30% hydrogen peroxide on a hot plate until it formed a white ash. The white ash was dissolved in 0.2 mL of 1.0N HCl and diluted with 0.8 mL deionized water. One mL of 0.5% ascorbic acid was added and the mixture was blended. After 20 min, 1 mL of 10% ammonium acetate buffer and 1 mL of 1 mM ferrozine color reagent were added and the mixture was mixed well. The mixture was allowed to stand at room temperature for 45 min before absorbance was determined at 562 nm. Tissue nonheme iron was extracted with 0.5 mL of 25% trichloroacetic acid and 0.5 mL of 4% pyrophosphate (Foy et al., 1967). These were added to 0.8 mL of 20% tissue homogenate and boiled in a waterbath for 20 min. The mixture was then centrifuged at 4,000 × g for 5 min, and the supernatant was retained. Extraction was done three times on each sample. One mL of combined supernatant was used for determining nonheme iron by the ferrozine method.

RESULTS & DISCUSSION

Iron content

Muscle tissue contains considerable iron bound to proteins. Myoglobin is the most abundant hemoprotein in muscle tissue. Total iron content of beef round muscle was 24.10 ± 1.15 μg/g wet weight, and nonheme iron content was 7.53 ± 0.23 μg/g wet weight. The proportion of nonheme iron to total iron was 31.2%.

Effect of homogenizing buffers

We determined the inhibitory effect of phytic acid in beef homogenates with several buffers including Tris, Hepes, Pipes, phosphate, and NaCl at pH 6.5 on the formation of TBARS as an indication of lipid peroxidation (Table 1). In the homogenates with all homogenizing buffers, 2 mM of phytic acid inhibited TBARS formation. The inhibition effect of phytic acid on lipid peroxidation as well as the TBARS value was highest in homogenates with phosphate buffer. Phytic acid also inhibited lipid peroxidation well in Hepes and Pipes buffers but had a low inhibitory effect when used in NaCl or Tris buffer. The TBARS value was lowest with Tris buffer.

Tris, Hepes, Pipes, phosphate, and NaCl buffers have been used in studies of metal-catalyzed oxidation, and they may affect experimental data by chelating metals or changing their redox state, ultimately resulting in Fe^{2+} autoxidation (Miller et al., 1990). Phytic acid is also known to accelerate autoxidation of Fe^{2+} to Fe^{3+} (Graf and Eaton, 1990) and to form a unique iron chelate (Graf et al., 1987). Thus, actions of phytic acid may be complicated by the use of such buffers in metal-catalyzing lipid peroxidation studies. Several types of phosphates inhibit lipid peroxidation and prevent warmed-over flavor (WOF) in beef and turkey (Akamittath et al., 1990; Trout and Dale, 1990). Such actions induced by high doses of phosphates may be due either to action of chelators of free metals (Tims and Watts, 1958), to shifting the ratio of Fe^{2+}/Fe^{3+} (Aust et al., 1985) and/or to increasing pH (Miller et al., 1986). However, in our results the formation of TBARS in the beef homogenates with the phosphate buffer was increased over that in the other buffers. The small amount of phosphate (50 mM) might provide an appropriate environment for maximum lipid peroxidation by maintaining the ratio of Fe^{2+}/Fe^{3+} at 1:1 (Aust et al., 1985).

Tris, Hepes and Pipes can also affect metal-catalyzed reactions by forming metal complexes. They seem to have their most pronounced effects in conjunction with other weak metal chelators. Tris inhibited lipid peroxidation in a system without Fe^{3+} chelators (Miller et al., 1990). NaCl oxidizes myoglobin to metmyoglobin via an anion-promoted autoxidation and eventually increases lipid peroxidation (Wallace et al., 1982). In addition, NaCl seems to displace iron ions from binding sites and thereby affect nonheme iron-dependent muscle lipid peroxidation (Kanner et al., 1991a). Although the mechanism of the antioxidant action of phytic acid on such homogenizing buffers was not elucidated in our study, the extent of the effect of phytic acid varied among buffers. Thus, buffers for such studies should be carefully selected.

Influence of pH

The ability of 2 mM phytic acid to inhibit lipid peroxidation at various pHs was determined (Table 2). As pH increased, TBARS formation increased in homogenates with 50 mM Hepes buffer and the inhibition by phytic acid also increased with pH. Metal-phytate complexes are highly insoluble over a wide pH range (Graf and Eaton, 1990). The action of phytic acid may inhibit metal-catalyzed lipid peroxidation over a wide pH range. Our results showed that the antioxidant effects of phytic acid were much higher at neutral or higher pH.

Nonheme iron-catalyzed oxidation is pH-sensitive and is most active in acidic pH; the optimum pH is 5.5. No catalysis occurs above pH 6.4 (Wills, 1965). At low pH levels, no matter how much phytic acid is present, iron would be available to participate in lipid peroxidation. On the other hand, hemoprotein-catalyzed oxidation is most active at alkaline pH (Lee et al., 1975). Methemoglobin accelerates linoleic acid peroxidation at pH from 5.6 to 7.8; catalysis is especially rapid at higher pH. In a model system of linoleic acid emulsion, the oxygen uptake rate by a chicken homogenate in phosphate buffer increased with pH; uptake at pH 7.5 was about two times greater than at pH <7.0, indicating that hemoprotein catalyzed lipid peroxidation (Lee et al., 1975). Catalysis of lipid peroxidation by ascorbate and ferritin-containing fraction from beef increased slightly with increasing pH from 5.0 to 7.0 (Seman et al., 1991). Our results with beef homogenate seemed to be characteristic of hemoprotein-catalyzed lipid peroxidation. However, many reducing or oxidizing factors in beef muscle might affect lipid peroxidation at different pH levels. At high pH the inhibitory effect of phytic acid may be due to its binding with iron to form a complex that may be unavailable to participate in lipid peroxidation.

Table 1—Effect of phytic acid (2 mM) on lipid peroxidation in beef homogenate with varying buffers (50 mM) at pH 6.5[a]

Buffers[b]	TBARS (nmole/g sample)	Inhibition (%)
Tris		
Control	7.92 ± 0.19	
Phytic acid	5.92 ± 0.31	25.3
HEPES		
Control	10.29 ± 0.14	
Phytic acid	4.30 ± 0.14	58.2
PIPES		
Control	12.08 ± 0.38	
Phytic acid	5.76 ± 0.59	52.3
Phosphate		
Control	43.88 ± 1.00	
Phytic acid	17.52 ± 0.14	60.1
NaCl		
Control	8.86 ± 0.27	
Phytic acid	6.92 ± 0.19	21.9

[a] Data represent the mean ± SD of three determinations.
[b] Tris: tris (hydroxymethyl) aminomethane, HEPES: N-2-hydroxyethyl piperazine-N'-2-ethanesulfonic acid, PIPES: piperazine-N,N'-bis(2-ethanesulfonic acid), Phosphate: sodium phosphate (dibasic anhydrous).

Table 2—Effect of phytic acid (2 mM) on lipid peroxidation in beef homogenate with 50 mM Hepes buffer at varying pH[a]

pH of homogenate	TBARS (nmole/g sample)		Inhibition (%)
	Control	Phytic acid	
5.0	3.46 ± 0.43	2.40 ± 0.19	30.6
6.0	8.40 ± 0.39	5.05 ± 0.43	39.9
7.0	26.88 ± 0.38	6.48 ± 0.44	75.9
8.0	30.39 ± 0.43	5.95 ± 0.55	79.4

[a] Data represent the mean ± SD of three determinations.

Iron-induced lipid peroxidation

Addition of 10 ppm iron ($FeCl_3$) to beef homogenate with 50 mM Hepes buffer at pH 7.0 enhanced lipid peroxidation (Fig. 1). However, the simultaneous addition of phytic acid reduced TBARS to a level lower than in the control. The most important catalysts of muscle lipid peroxidation appear to be free metal ions, of which iron is probably the most active. Storing frozen-thawed muscle, cooking, and adding of ascorbic acid or hydrogen peroxide to beef increase the amount of low-molecular-weight iron (Decker and Hultin, 1990; Kanner et al., 1991b). Baking and microwaving ground beef increases the concentration of nonheme iron, as does the addition of ascorbic acid and hydrogen peroxides (Schricker and Miller, 1983). The amount of ascorbate-induced iron released from ferritin decreases as pH increases. Temperature also affects the release of iron from ferritin. Physiological concentrations of heated and

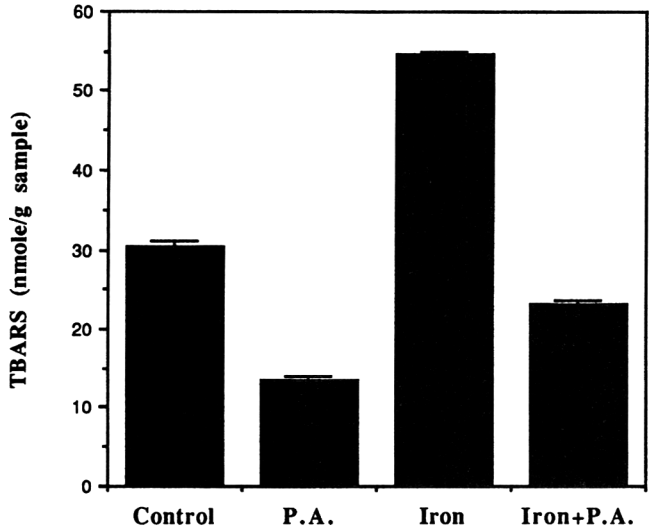

Fig. 1—Effect of 1 mM phytic acid (P.A.) and 10 ppm iron ($FeCl_3$) on lipid peroxidation in beef homogenate with 50 mM Hepes buffer at pH 7.0.

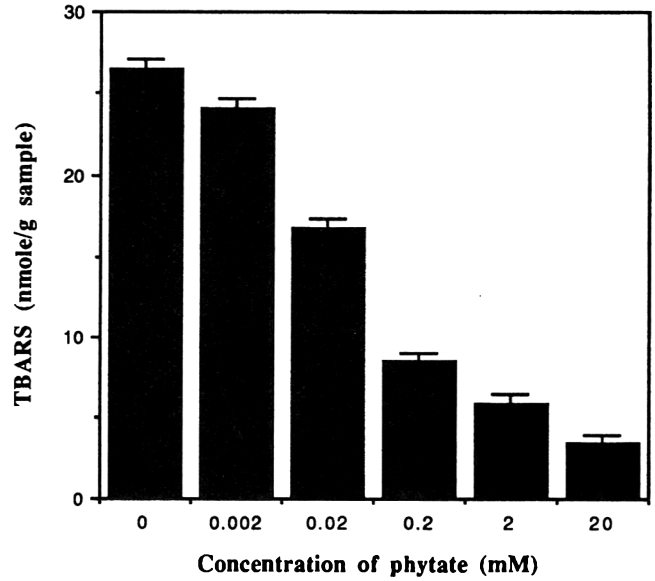

Fig. 2—Effect of phytic acid on lipid peroxidation in beef homogenate with 50 mM Hepes buffer at pH 7.0.

uncooked ferritin can catalyze lipid peroxidation of beef in the presence of ascorbic acid or cysteine, promoting the development of rancidity and WOF (Decker and Welch, 1990).

In our study, the added ferric ion could produce lipid peroxyl radicals by decomposing the lipid hydroperoxides preformed in the muscle food or formed in the process of homogenation, which could stimulate the chain reaction of lipid peroxidation by extracting further hydrogen atoms (Schaich, 1992). In addition, many reducing factors in muscle foods such as superoxides, ascorbates, glutathione and cysteine may affect the reduction of added ferric to ferrous ion which can initiate lipid peroxidation via the iron-catalyzed Haber-Weiss reaction (Halliwell and Chirico, 1993; Minotti and Aust, 1992). The action of phytic acid may be due to maintaining the ferric ion state and chelating with ferric ion, which inhibits the initiation and propagation of lipid peroxidation.

Concentration-dependent effect

We measured the ability of phytic acid (0 to 20 mM) to inhibit lipid peroxidation (Fig. 2). Phytic acid inhibited formation of TBARS in a dose-dependent manner. At 20 mM of phytic acid, inhibition was 86.9%. Iron bound to negatively charged phospholipids promotes lipid peroxidation and consequently generates WOF (Graf and Panter, 1991). Iron chelation by phytic acid reduced formation of WOF. Phytic acid exhibits a high affinity for polyvalent cations and forms water-excluding chelates (Graf et al., 1984). One phytate molecule can bind up to six divalent cations and the metal might bridge two or more phytate molecules, depending on the redox state (Graf and Eaton, 1990). When all six coordination sites in Fe^{3+}-phytate chelate are occupied, the chelate cannot participate in the Fenton reaction. This explains how phytic acid inhibits ·OH generation and subsequent lipid peroxidation (Graf et al., 1984). In molar ratios of 0.25 phytate-to-iron and above the superoxide-driven generation of hydroxyl radicals is almost completely blocked (Graf and Eaton, 1990). Mathematically if 0.135 mM (7.54 mg/kg) of nonheme iron was in the beef, 0.034 mM of phytate or more would block completely the hydroxyl radical production. The antioxidant effect of phytic acid was largely due to its ferroxidase activity (Graf et al., 1987). Phytic acid accelerates the oxidation of Fe^{2+}, but does not affect reduction of Fe^{3+}.

Comparison with other antioxidants

Commercially used food antioxidants including ascorbic acid, BHT, and EDTA were compared with phytic acid to determine

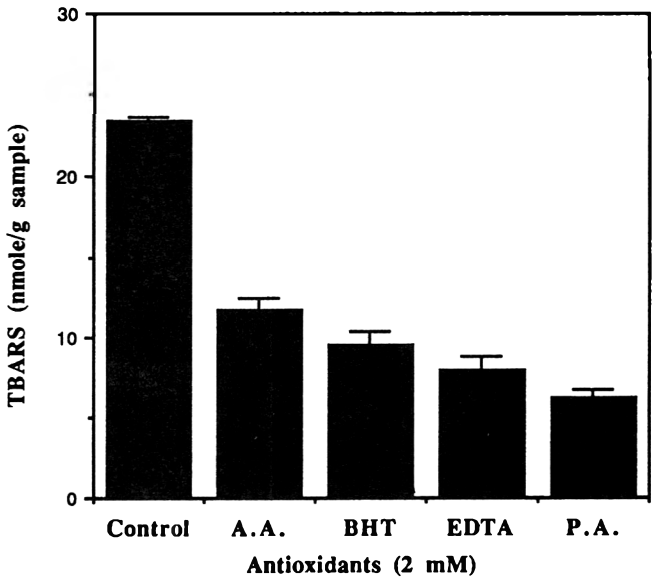

Fig. 3—Effect of antioxidants (2 mM) on lipid peroxidation in beef homogenate with 50 mM Hepes buffer at pH 7.0. A.A. = ascorbic acid; BHT = butylated hydroxy-toluene; EDTA = ethylene diaminetetraacetic acid; P.A. = phytic acid.

lipid peroxidation inhibition in beef homogenates (Fig. 3). Phytic acid inhibited TBARS formation by 73.7%, more than the other antioxidants (50.2% for ascorbic acid, 59.2% for BHT and 66.3% for EDTA). When meat is cooked, iron liberated from myoglobin interacts with phospholipids to catalyze lipid peroxidation, thereby increasing the development of WOF. This Fe^{3+}-phospholipid complex increases TBARS by catalyzing the rapid autoxidation of its unsaturated fatty acyl moieties. Both EDTA and ascorbate can be antioxidant and prooxidant depending on iron concentration and degree of hydrophobicity of substrate microenvironment (Mahoney and Graf, 1986). The antioxidant effect of EDTA may be due to the inhibition of nonheme iron activity. However, EDTA did not affect the oxidation catalyzed by hemoproteins such as hemoglobin or cytochrome.

The ability of ascorbic acid to inhibit oxidation is probably due to its ability to keep the heme pigment in the inactive ferrous state. Harel et al. (1988) found that EDTA at the relatively low concentration of 20–25 μM inhibits redox-cycle membrane

lipid peroxidation. Free metal ions increase lipid peroxidation in minced turkey muscle and EDTA inhibits lipid peroxidation in both raw and heated turkey muscle (Kanner et al., 1988b). Vitamin C treatment in raw ground beef showed low pigment and lipid peroxidation (Mitsumoto et al., 1991). Ascorbic acid inhibits lipid peroxidation in a concentration-dependent manner. Low levels of ascorbic acid promote WOF caused by lipid peroxidation. High concentrations of ascorbic acid inhibit WOF as determined by the TBA test (Fischer and Deng, 1977; Sato and Hegarty, 1971). Ascorbic acid accelerates iron-catalyzed lipid peroxidation, while it delays the hemoglobin-catalyzed lipid peroxidation (Wills, 1965). Addition of 100 ppm ascorbic acid to linoleate emulsions does not affect oxygen uptake at pH 5.6, but strongly inhibits uptake at pH 7.8 (Fischer and Deng, 1977). Oxidation of ascorbic acid is reduced to baseline when 1 mM phytic acid is included in the Fe^{3+}-hydrogen peroxide samples (Empson et al., 1991).

Although phytic acid is not listed as GRAS (generally recognized as safe) by the Food and Drug Administration of the United States, it is extensively used as a food additive outside the U.S. The antioxidant or iron-chelating properties of phytic acid render this compound a unique and versatile food preservative. Phytic acid can be added to soybean oil and other lipid-containing foods to prevent both autoxidation and hydrolysis. Addition of phytic acid to meats, fish meal pastes, canned seafoods, fruits, and vegetables prevents both product discoloration and lipid peroxidation, resulting in improvement of the nutritional quality of many foods and prolonged shelf-life.

CONCLUSIONS

THE ADDITION OF SMALL AMOUNTS of phytic acid may inhibit lipid peroxidation both by accelerating autoxidation of ferrous to ferric ion and by forming catalytically inactive iron chelates. Phytic acid more effectively inhibits lipid peroxidation than other antioxidants such as ascorbate, BHT and EDTA in beef homogenates. Its antioxidant effect is concentration- and pH-dependent. Presumably phytic acid removes myoglobin-derived iron from negatively charged phospholipids and prevents their autoxidation and off-flavor formation. In stored or mixed foods such as chili con carne, phytic acid may protect against lipid decomposition by iron-catalyzed peroxidation.

REFERENCES

Akamittath, J.G., Brekke, C.J., and Schanus, E.G. 1990. Lipid peroxidation and color stability in restructured meat systems during frozen storage. J. Food Sci. 55: 1513–1517.

Aust, S.D., Morehouse, L.A., and Thomas, C.E. 1985. Role of metals in oxygen radical reactions. Free Radic. Biol. Med. 1: 3–25.

Buege, J.A. and Aust, S.D. 1978. Microsomal lipid peroxidation. Methods Enzymol. 52: 302–304.

Decker, E.A. and Welch, B. 1990. Role of ferritin as a lipid oxidation catalyst in muscle food. J. Agr. Food Chem. 38: 674–677.

Decker, E.A. and Hultin, H.O. 1990. Factors influencing catalysis of lipid oxidation by soluble fraction of mackerel muscle. J. Food Sci. 55: 947–950.

Ellis, R., Morris, E.R., and Hill, A.D. 1982. Bioavailability to rats of iron and zinc in calcium-iron-phytate and calcium-zinc-phytate complexes. Nutr. Res. 2: 319–322.

Empson, K.L., Labuza, T.P., and Graf, E. 1991. Phytic acid as a food antioxidant. J. Food Sci. 56: 560–563.

Fischer, J. and Deng, J.C. 1977. Catalysis of lipid peroxidation. A study of mullet (*Mugi cehalus*) dark flesh and emulsion model systems. J. Food Sci. 42: 610–614.

Foy, A.L., Williams, H.R., Cortell, S., and Cornard, M.E. 1967. A modified procedure for the determination of nonheme iron in tissue. Anal. Biochem. 18: 559–563.

Graf, E. and Eaton, J.W. 1985. Dietary suppression of colonic cancer. Fiber or phytate? Cancer 56: 717–718.

Graf, E. and Eaton, J.W. 1990. Antioxidant functions of phytic acid. Free Radic. Biol. Med. 8: 61–69.

Graf, E., Empson, K.L., and Eaton, J.W. 1987. Phytic acid. A natural antioxidant. J. Biol. Chem. 262: 11647–11650.

Graf, E., Mahoney, J.R., Bryant, R.G., and Eaton, J.W. 1984. Iron-catalyzed hydroxyl radical formation. Stringent requirement for free iron coordination site. J. Biol. Chem. 259: 3620–3624.

Graf, E. and Panter, S.S. 1991. Inhibition of warmed-over-flavor development by polyvalent cations. J. Food Sci. 56: 1055–1058.

Halliwell, B. and Chirico, S. 1993. Lipid peroxidation: its mechanism, measurement and significance. Am. J. Clin. Nutr. 57(Suppl): 715s–725s.

Halliwell, B. and Gutteridge, J.M.C. 1986. Oxygen free radicals and iron in relation to biology and medicine. Some problems and concepts. Arch. Biochem. Biophys. 246: 501–514.

Harel, S., Shegalovich, J., Hazan, B., and Kanner, J. 1988. Lipid peroxidation-dependent on oxygen and catalytic free iron ions in situ storage muscle food. In *Oxygen Radicals in Biology and Medicine*, M.G. Simic et al. (Ed.), p. 301–306. Plenum Press, New York.

Jariwalla, R.J., Sabin, R., Lawson, S., Bloch, D.A., Prender, M., Andrews, V., and Herman, Z.S. 1988. Effects of dietary phytic acid (phytate) on the incidence and growth rate of tumors promoted in Fisher rats by a magnesium supplement. Nutr. Res. 8: 813–827.

Kanner, J., Harel, S., and Jaffe, R. 1991a. Lipid peroxidation of muscle food as affected by NaCl. J. Agric. Food Chem. 39: 1017–1021.

Kanner, J., Hazan, B., and Doll, L. 1988a. Catalytic "free" iron ions in muscle foods. J. Agr. Food Chem. 36: 412–415.

Kanner, J., Salan, M.V., Harel, S., and Shegalovich, J. 1991b. Lipid peroxidation of muscle food: The role of the cytosolic fraction. J. Agr. Food Chem. 39: 242–246.

Kanner, J., Shegalovich, I., Harel. S., and Hazan, B. 1988b. Muscle lipid peroxidation dependent on oxygen and free metal ions. J. Agr. Food Chem. 36: 409–417.

Keller, J.D. and Kinsella, J.E. 1973. Phospholipid changes and lipid oxidation during cooking and frozen storage of raw ground beef. J. Food Sci. 38: 1200–1204.

Khayat, A. and Schwall, D. 1983. Lipid oxidation in sea food. Food Technol. 37(7): 130–140.

Kubow, S. 1992. Routes of formation and toxic consequences of lipid peroxidation products in foods. Free Radic. Biol. Med. 12: 63–81.

Kwoh, T.L. 1971. Catalysts of lipid peroxidation in meats. J. Am. Oil Chem. 48: 550–555.

Lee, Y.B., Hargus, G.L., Kirkpatrick, J.A., Bemmer, D.L., and Forsythe, R.H. 1975. Mechanism of lipid peroxidation in mechanically deboned chicken meat. J. Food Sci. 40: 964–967.

Love, J.D. 1983. The role of heme iron in oxidation of lipids in red meats. Food Technol. 37(7): 118–120.

Love, J.D. and Pearson, A.M. 1974. Metmyoglobin and nonheme iron as prooxidants in cooked meat. J. Agr. Food Chem. 22: 1032–1034.

Mahoney, J.R. and Graf, E. 1986. Role of alpha-tocopherol, ascorbic acid, citric acid and EDTA as oxidants in model systems. J. Food Sci. 51: 1293–1296.

Miller, D.M., Buettner, G.R., and Aust, S.D. 1990. Transition metals as catalyst of "autoxidation" reactions. Free Radic. Biol. Med. 8: 95–108.

Miller, M.G., David, G.W., Seidemann, S.C., Ramsey, C.B., and Rolan, T.L. 1986. Effects of various phosphates on the palatability, appearance and storage traits of flaked and formed restructured beef steaks. J. Food Sci. 51: 1435–1438.

Minotti, G. and Aust, S.D. 1992. Redox cycling of iron and lipid peroxidation. Lipids 27: 219–226.

Mitsumoto, M., Faustman, C., Cassens, R.G., Arnold, R.N., Schaefer, D.M., and Scheller, K.K. 1991. Vitamins E and C improve pigment and lipid stability in ground beef. J. Food Sci. 56: 194–197.

Pearson, A.M., Gray, J.I., Arlene, M., and Horenstein, N.A. 1983. Safety implication of oxidized lipids in muscle food. Food Technol. 37(7): 121–129.

Sato, K. and Hegarty, G.R. 1971. Warm-over flavor in cooked meats. J. Food Sci. 36: 1098–1102.

Schaich, K.M. 1992. Metals and lipid peroxidation. Contemporary issue. Lipids 27: 209–218.

Schricker, B.R. and Miller, D.D. 1983. Effect of cooking and chemical treatment on heme and nonheme iron in meat. J. Food Sci. 48: 1340–1342.

Seman, D.L., Decker, E.A., and Crum, A.D. 1991. Factors affecting catalysis of lipid peroxidation by a ferritin-containing extract of beef muscle. J. Food Sci. 56: 356–358.

Shamsuddin, A.M., Elslayed, A.M., and Ullah, A. 1988. Suppression of large intestinal cancer in F344 rats by inositol hexaphosphate. Carcinogenesis 9: 577–580.

Stookey, L.L. 1970. Ferrozine-a new spectrophotometric reagent for iron. Anal. Chem. 42: 779–781.

Tims, M.J. and Watts, B.M. 1958. Protection of cooked meats with phosphates. Food Technol. 12: 240–243.

Trout, G.R. and Dale, S. 1990. Prevention of warmed-over flavor in cooked beef: Effect of phosphate type, phosphate concentration, a lemon juice/phosphate blend, and beef extract. J. Agric. Food Chem. 38: 665–669.

Wallace, W.J., Houtchens, R.A., Maxwell, J.C., and Caughey, W.S. 1982. Mechanism of autooxidation for hemoglobins and myoglobins. Promotion of superoxide production by proteins and anions. J. Biol. Chem. 257: 4966–4977.

Wills, E.D. 1965. Mechanisms of lipid peroxide formation in tissue: Role of metals and hematin proteins in the catalysis of oxidation of unsaturated fatty acids. Biochim. Biophys. Acta 98: 238–251.

Wilson, B.R., Pearson, A.M., and Shorland, F.B. 1976. Effect of total lipids and phospholipids on warmed-over flavor in red muscle and white muscle from several species as measured by the thiobarbituric acid analysis. J. Agr. Food Chem. 24: 7–11.

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Flaked Sinew Addition to Low-fat Cooked Salami

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ABSTRACT

Flaked sinew (FS) was added at three levels (7.5, 15, and 22.5%) to low-fat, 20% added water (AW) salamis. A 1.27 cm Comitrol machine head was used to produce large (flaked once) and small (flaked twice) particle sizes. Treatments were preblended (P) and non-preblended (N). P and N controls contained 10% and 20% fat, respectively. Addition of FS decreased ($P < 0.05$) moisture compared to controls. Yields decreased ($P < 0.05$) as FS increased regardless of particle size; however, FS addition minimized purge. Lightness increased ($P < 0.05$) as FS increased in N treatments compared to the low-fat control. Break force/cm^2 increased ($P < 0.05$) in P controls compared to N controls. Large FS particle size N treatments had higher ($P < 0.05$) peak force values than those with small particle size. FS treatments were comparable to high-fat controls in palatability traits.

Key Words: beef, sinew, texture, salami, low-fat

INTRODUCTION

PROCESSED MEATS account for about 30% of U.S. meat production. Concern over calorie and salt intakes has resulted in a general reduction of meat consumption. New processes are being used to produce lower fat products, while attempting to minimize any resulting undesirable characteristics. Connective tissue proteins from hide and meat trimmings have been used as texture-modifying ingredients in many products such as hamburger (Chavez et al., 1985; Hunt and Campbell, 1992); emulsified sausages (Saffle et al., 1964; Jones et al., 1982a, b); and restructured meats (Liu et al., 1990; Kenney et al., 1992).

Desinewing technology has enabled removal of heavy connective tissue from less tender meat cuts to increase value and improve texture (Cross et al., 1978). This has produced a by-product, connective tissue trimmings, which could be modified and added to meat products. Particle size reduction permits production of flaked sinew (FS) of different particle sizes, which could make this by-product easier to handle and mix with other meat sources.

Preblending could be used to maximize the functional contribution of myofibrillar proteins (which are the most functional) when some would be replaced by lower quality proteins like connective tissue. Preblending the myofibrillar (salt-soluble protein) portion would allow more time for proteins to react with salt, phosphate, and water and improve functional characteristics (Hand et al., 1987; Acton and Saffle, 1969; Ockerman and Crespo, 1982).

Our objective was to evaluate the effect of flaked sinew (FS), at different levels and particle sizes, as well as the effect of preblending on chemical, color, storage, texture, and sensory characteristics of low-fat beef salami.

MATERIALS & METHODS

SHANKS FROM A-MATURITY CATTLE were desinewed using a Baader Desinewer (Model 699) at the IBP plant in Emporia, KS. The sinew was frozen (−20°C), tempered to 0°C, and initially flaked, using an Urschel® comitrol machine (1.91 cm comitrol head No. 2-J-030750). Then a 1.27 cm head (2-K-020050) was used to produce large (L) and small (S) particle sizes by passing the sinew through the Comitrol one or two times, respectively (Fig. 1). Temperature of flaked material never increased above 2°C. The FS was mixed; sampled for moisture, fat, and protein (AOAC, 1984); vacuum packaged in 1.2 kg bags; and frozen until processing. Batches of 4.55 kg were formulated with the Least Cost Formulator (Ldt., Inc., Virginia Beach, VA).

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Product manufacture

A processing scheme for manufacture of low- and high-fat salamis was used (Fig. 2). Lean beef and beef fat were thawed for 36 hr at 4°C, ground (first through a 1.27 cm plate and then a 0.32 cm plate), and mixed with appropriate ingredients. The control salamis contained 10% or 20% fat and 20% added water (AW) and were non-preblended (N) or preblended (P) for 12 hr at 4°C after mixing for 4 min in a Hobart mixer with 2.3% salt (includes salt contained in the prague powder), 0.25% prague powder (6.02% sodium nitrite), 0.3% tetrasodium phosphate, 0.05% sodium erythorbate, and 50% of the water. After preblending, the lean was mixed with the rest of the water; beef fat; and spices (0.03% nutmeg, 0.28% black pepper, 0.12% coriander, and 0.03% allspice). The meat block for all sinew-containing treatments was replaced partially with 7.5, 15, or 22.5% FS at either the L or S particle size and formulated to contain 10% fat and 20% (AW), P or N. A total of 48 treatments was evaluated during 3 days (replicates) of experimental product manufacture (16 treatments/day). The raw mixture was stuffed into a 6.35-cm-diameter fibrous casing using a piston stuffer (Frey, Model Compacter 1). The product was clipped with a Poly-clip® (Niedecker Gbmh & Co., West Germany), weighed, and heat processed in a Maurer smokehouse (Rauch und Warmetechnik Gmbh & Co., West Germany) for 30 min at 32°C, 30 min at 41°C, and 20 min at 49°C; smoked for 30 min; then cooked 10 min at 54°C; and smoked again for 10 min at 60°C. Finally, the temperature was elevated and held at 72°C and 50% relative humidity until the product reached an internal temperature of 68°C. The product was showered with water for 20 min and chilled for 12 hr. Product temperatures were monitored with thermocouples and a Doric Minitrend Data Logger (Model 205B-1-C-OTF).

Pumpability and pH

Raw product pumpability (kgf*mn) was measured, immediately after product mixing and after holding the product at 1.5°C for 24 hr, by using an Instron (Model 4201) attachment to the Instron Universal machine (Claus et al., 1990) in the 20% range of a 500 kg compression load cell (100 kg) and a crosshead speed of 200 mm/min. The pHs of raw and final products were measured by blending 10g of sample with 100 mL distilled water and using a Fisher Accumet pH Meter (Model 620) and Orion combination electrode. Measurements were repeated twice for each treatment.

Cooking yield and purge loss

After the salamis were chilled for 12 hr, they were reweighed, and the smokehouse cooking yield was calculated. Purge during storage was determined by placing eight slices (≈0.14 kg/package) into each of packages, which were weighed; vacuum packaged (SuperVac®, Smith Equipment Co., Clifton, N.J. 07012) in 20.32 × 30.48 cm laminated Cryovac bags; and stored for 1 week at 4°C. Also, two chubs/treatment were weighed (≈0.18 kg), vacuum packaged, and stored for 4 wk at 4°C. Purge loss for both slices and chubs was determined by draining liquid

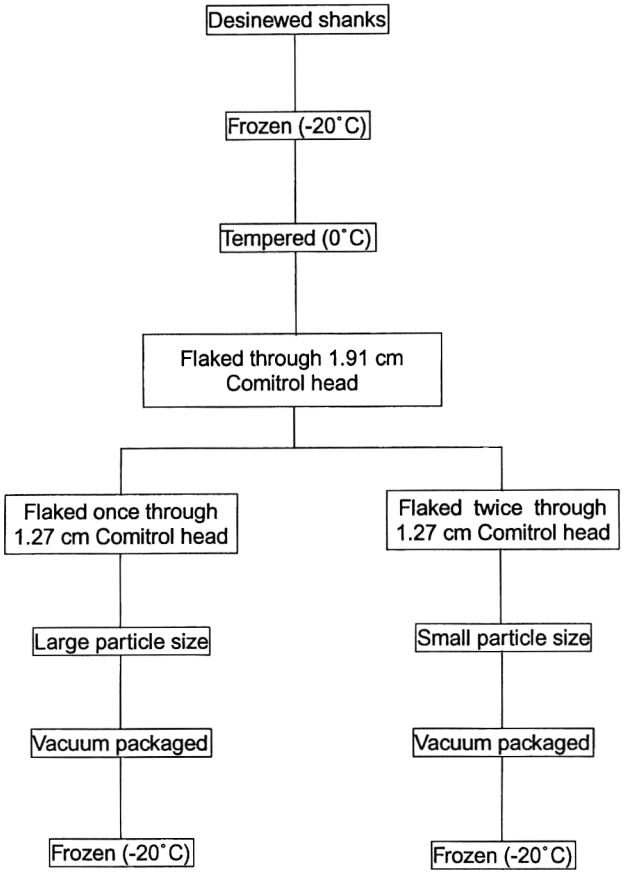

Fig. 1—Processing scheme for manufacture of large and small flaked sinew.

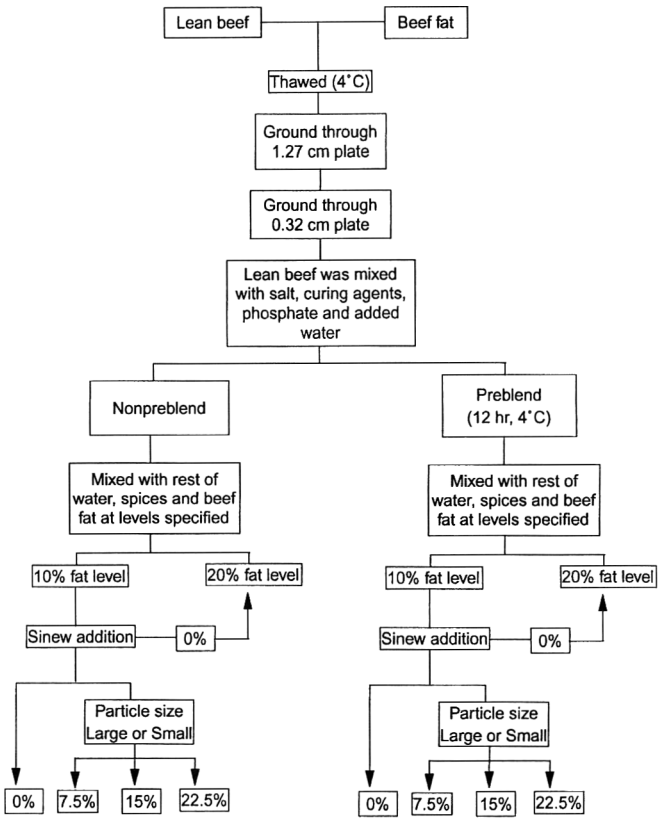

Fig. 2—Processing scheme for manufacture of low- and high-fat salami.

from the bags for 25 sec into preweighed test tubes and calculating percentage purge.

Chemical and color evaluations

Cooked product was analyzed for moisture (oven drying), fat (Foss-Let Fat Analyzer), and protein (AOAC, 1984). A Hunter Lab D54 Spectrophotometer was used to measure cured color development of the cooked salami. CIE L*, a*, and b* values for Illuminant A and C were determined by the average of 2 measurements. The second reading was taken after rotating the product 90° clockwise (Hunt et al., 1992). Also, percent reflectance was measured at 650 and 570 nm, and the ratio of those reflectance values was used to determine cured color development (Erdman and Watts, 1957).

Texture

After 10 days of vacuum storage at 4°C, texture profile analysis procedures developed by Bourne (1978) and Montejano et al. (1985) were followed. A core of beef salami (2.5 cm diameter, 2.1 cm high) was compressed axially to 75% of its height in order to determine hardness (peak force of first compression curve), cohesiveness (ratio of area under second compression curve and the area under the first compression curve * 100), and springiness (base width of second compression divided by base width of first). The Instron was programmed for a load range of 25 kg (5% of a 500 kg compression load cell) and a crosshead speed of 200 mm/min.

Lee-Kramer shear values were measured on 5.5 × 5.5 × 1 cm samples of beef salami with the Instron Lee-Kramer cell utilizing a load range of 25 kg (5% of a 500-kg load cell) and a crosshead speed of 350 mm/min. Peak force (kgf) and total energy (kgf*mm) were determined and divided by the weight of each sample to determine force or energy/g product. Tensile strength was measured using a range of 2.5 kg (10% of a 50 kg tension load cell) and a crosshead speed of 200 mm/min. Each end of a 1-cm-thick, 3-cm-wide strip was attached to pneumatic grips via metal pins embedded in two metal plates, which were mounted on crosshead pneumatic grips. Peak, break force, and total energy were recorded. All texture measurements were repeated 3 times for each treatment.

Sensory analysis

A trained, five-member, sensory panel from the Kansas State University Sensory Center was used to analyze beef salami for palatability characteristics after 10 days of vacuum storage at 4°C. Trained panelists had completed 120 hr of training in flavor and texture analysis, had completed a minimum of 1,000 hr of general sensory testing, and had experience on flavor and texture descriptive analysis in meat and meat products. All panelists received 2 hr of orientation appropriate to this study. Panel training consisted of panel members individually examining a product representative of that under consideration (references). Scores for each attribute were designated by the panelists using a 15-point scale and placing the appropriate number on the score sheet. Slices of apple, unsalted crackers, and water were given between samples. Sensory sessions were held under red lights once a day for 4 days, until all treatments were evaluated.

From each treatment a 1-cm-thick slice (5.5 cm diameter) cut in half was presented to each panelist. They evaluated completeness of shear (0 = small residue to 15 = large amount of residue), deformation (0 = little deformation to 15 = large deformation prior to breaking), cohesiveness of mass (0 = distinct particles to 15 = mass of particles), firmness (0 = little force to 15 = high amount of force), springiness (0 = small return to original shape to 15 = high return to original shape), difficulty of mastication (0 = little force to chew sample to 15 = large amount of force required to chew the sample), moisture (0 = small amount of liquid to 15 = large amount of liquid), connective tissue residue (0 = small amount of residual tissue to 15 = large amount of residual tissue), connective tissue size (0 = small particles to 15 = large particles), spice impact (0 = small amount of aromatic to 15 = large amount of aromatic), saltiness (0 = small amount of sodium chloride to 15 = large amount of sodium chloride), and astringency (0 = little puckering sensation to 15 = high puckering sensation).

Statistical analysis

Statistical analysis (SAS Institute, Inc., 1992) determined treatment effect from 16 treatments and three replications by using a blocked one-way analysis of variance. Various hypotheses were tested, comparing means of treatment combinations and simultaneous tests of interaction between FS particle size, and added FS level; preblending and FS level; preblending, FS particle size and FS level; preblending and FS particle

Table 1—Chemical composition of nonpreblended (N) and preblended (P) cooked salamis with or without large (L) and small (S) particle sizes of flaked sinew (FS) (means and standard error)

Treatments[hi]	% Moisture	% Fat	% Protein[j]	% AW[k]
NControl 10%	70.18[a]	11.38[e]	12.78[b]	19.05[bc]
NControl 20%	61.14[e]	21.28[a]	10.40[cde]	19.53[bc]
NL 7.5% FS	67.36[cd]	13.82[bc]	8.46[g]	33.52[a]
NL 15% FS	68.39[bc]	12.50[cde]	11.52[bc]	22.32[b]
NL 22.5% FS	67.64[bc]	13.46[cd]	19.07[a]	−8.64[d]
NS 7.5% FS	67.51[bcd]	13.63[c]	9.18[efg]	30.80[a]
NS 15% FS	67.48[bcd]	13.67[bc]	12.64[b]	16.92[bc]
NS 22.5% FS	68.21[bc]	12.74[cde]	19.46[a]	−9.63[d]
PControl 10%	68.81[b]	11.97[de]	12.37[b]	19.32[bc]
PControl 20%	61.90[e]	19.70[a]	10.61[cd]	18.95[bc]
PL 7.5% FS	68.00[bc]	13.01[cd]	9.82[def]	28.73[a]
PL 15% FS	67.27[cd]	13.94[bc]	12.64[b]	16.71[bc]
PL 22.5% FS	68.38[bc]	12.53[cde]	19.44[a]	−9.04[d]
PS 7.5% FS	67.91[bc]	13.13[cd]	8.70[fg]	33.10[a]
PS 15% FS	67.26[cd]	13.63[c]	12.78[b]	16.49[c]
PS 22.5% FS	66.25[d]	15.25[b]	18.79[a]	−8.90[d]
SE	0.48	0.57	0.47	2.01

[a-g] Means in the same column with same superscript letter do not differ ($P < 0.05$).
[h] Control treatments were formulated to contain 10% or 20% fat.
[i] The percent value indicated on all FS added treatments represents sinew content.
[j] Protein values: 6.25, 6.20, and 6.15 for 7.5%, 15%, and 22.5% FS treatments, respectively, and 5.97 for the control.
[k] AW = % moisture − 4 times the protein.

Table 2—Cooked yield, pH, and purge loss of nonpreblended (N) and preblended (P) salamis with or without large (L) and small (S) particle sizes of flaked sinew (FS) (means and standard error)

			% Purge	
Treatments[gh]	% Yield	PH cooked	Slices	Chubs
NControl 10%	89.78[ab]	6.32[b]	1.33[a]	0.78[a]
NControl 20%	91.33[a]	6.42[ab]	0.83[b]	0.50[b]
NL 7.5% FS	87.55[cd]	6.41[ab]	0.31[c]	0.00[e]
NL 15% FS	86.20[de]	6.46[a]	0.00[d]	0.00[e]
NL 22.5% FS	85.63[ef]	6.51[a]	0.00[d]	0.00[e]
NS 7.5% FS	87.25[cd]	6.42[ab]	0.00[d]	0.00[e]
NS 15% FS	87.43[cd]	6.50[a]	0.00[d]	0.00[e]
NS 22.5% FS	85.60[ef]	6.49[a]	0.00[d]	0.00[e]
PControl 10%	88.68[bc]	6.39[ab]	0.82[b]	0.24[c]
PControl 20%	90.48[a]	6.42[ab]	0.23[c]	0.10[d]
PL 7.5% FS	86.37[de]	6.41[ab]	0.00[d]	0.00[e]
PL 15% FS	86.17[de]	6.44[ab]	0.00[d]	0.00[e]
PL 22.5% FS	85.65[ef]	6.48[a]	0.00[d]	0.00[e]
PS 7.5% FS	86.02[de]	6.42[ab]	0.00[d]	0.00[e]
PS 15% FS	85.30[ef]	6.47[a]	0.00[d]	0.00[e]
PS 22.5% FS	84.40[f]	6.41[ab]	0.00[d]	0.00[e]
SE	0.57	0.05	0.07	0.03

[a-f] Means in the same column with same superscript letter do not differ ($P > 0.05$).
[g] Control treatments were formulated to contain 10% or 20% fat.
[h] The percent value indicated on all FS added treatments represents sinew content.

Table 3—Spectrophotometric color measurements of nonpreblended (N) and preblended (P) salamis with or without large (L) and small (S) particle sizes of flaked sinew (FS) (means and standard error)

	Illuminant C			
Treatments[hi]	L*	a*	b*	Ratio[j]
NControl 10%	50.38[e]	15.60[ab]	7.74[gh]	2.58[a]
NControl 20%	53.16[abcd]	13.34[g]	7.61[h]	2.31[defg]
NL 7.5% FS	52.00[d]	14.04[fg]	8.09[efgh]	2.42[bcd]
NL 15% FS	52.56[cd]	14.94[bcd]	8.81[abc]	2.44[bcd]
NL 22.5% FS	53.63[abc]	14.08[efg]	8.96[ab]	2.27[abc]
NS 7.5% FS	52.20[d]	15.37[abc]	8.31[cdef]	2.49[ab]
NS 15% FS	53.04[abcd]	14.71[cdef]	8.57[bcde]	2.40[bcde]
NS 22.5% FS	53.81[abc]	14.40[efg]	9.22[a]	2.34[cdef]
PControl 10%	52.14[a]	15.92[a]	7.97[fgh]	2.57[a]
PControl 20%	54.01[ab]	14.39[def]	8.34[cdef]	2.32[cdefg]
PL 7.5% FS	52.68[bcd]	15.09[abcd]	8.18[defg]	2.44[bcd]
PL 15% FS	52.84[abcd]	14.52[cdef]	8.35[cdef]	2.37[bcdef]
PL 22.5% FS	53.37[abcd]	14.55[cdef]	8.69[abcd]	2.35[cdef]
PS 7.5% FS	52.72[abcd]	14.92[bcde]	8.06[efgh]	2.41[bcde]
PS 15% FS	54.13[a]	14.98[efg]	8.55[bcde]	2.29[efg]
PS 22.5% FS	53.90[abc]	13.40[g]	9.02[ab]	2.21[g]
SE	0.50	0.30	0.20	0.05

[a-g] Means in the same column with same superscript letter do not differ ($P > 0.05$).
[h] Control treatments were formulated to contain 10% or 20% fat.
[i] The percent value indicated on all FS added treatments represents sinew content.
[j] %R650/%R570 nm.

Table 4—Instron texture profile analysis of nonpreblended (N) and preblended (P) salamis with or without large (L) and small (S) particle sizes of flaked sinew (FS) (means and standard error)

Treatments[gh]	Cohesiveness[i]	Hardness[j]	Springiness[k]
NControl 10%	36.42[b]	33.63[abc]	4.00[f]
NControl 20%	28.11[b]	35.83[a]	4.89[bcdef]
NL 7.5% FS	37.86[b]	33.78[abc]	4.33[ef]
NL 15% FS	34.29[b]	34.98[ab]	4.78[cdef]
NL 22.5% FS	41.94[b]	21.36[e]	6.11[a]
NS 7.5% FS	43.26[b]	23.91[de]	5.67[abc]
NS 15% FS	32.40[b]	25.60[bcde]	5.56[abcd]
NS 22.5% FS	74.78[a]	24.51[cde]	5.00[bcdef]
PControl 10%	47.65[b]	26.87[abcde]	5.22[abcde]
PControl 20%	41.94[b]	23.35[de]	5.89[ab]
PL 7.5% FS	48.52[b]	26.30[abcde]	5.44[abcd]
PL 15% FS	39.50[b]	32.39[abcd]	4.11[f]
PL 22.5% FS	37.59[b]	33.61[abc]	4.33[ef]
PS 7.5% FS	43.23[b]	28.95[abcde]	4.56[def]
PS 15% FS	36.17[b]	32.62[abcd]	4.22[ef]
PS 22.5% FS	29.64[b]	30.39[abcde]	4.22[ef]
SE	8.98	3.44	0.36

[a-f] Means in the same column with same superscript letter do not differ ($P > 0.05$).
[g] Control treatments were formulated to contain 10% or 20% fat.
[h] The percent value indicated on all FS added treatments represents sinew content.
[i] (A1/A2)* 100
[j] Kgf
[k] (B1/B2)

size; P and N controls; low- and high-fat controls; and preblending and non-preblending.

RESULTS & DISCUSSION

Chemical composition

Moisture and fat contents of small FS particle size raw materials (moisture = 54.6%, fat = 17%) did not differ ($P > 0.05$) from large FS counterparts (moisture = 55.4%, fat = 18.9%). Chemical composition data (Table 1) showed that the N low-fat control was within 1.38% and 0.95%, respectively, of the formulated targets for fat (11%) and AW (20%), and the N high-fat control was within 1.28% and 0.47%, respectively, of the target amounts for fat and AW. The low-fat P controls were within 1.97% and 0.68% and the high-fat P controls were within 0.30% and 1.05% of the target levels of fat and AW, respectively. Protein content increased ($P < 0.05$) as added FS increased. FS treatments were significantly different ($P < 0.05$) in moisture from the N low-fat control. Preblending did not increase moisture content or AW level for the treatments. End-point internal temperature (data not shown) was the same ($P > 0.05$) among treatments but the treatments with added FS required longer to cook (about 2 more hr). This was probably because the FS reduced heat transfer within the sausage and increased moisture loss, which caused a cooling effect. Minimal casing distortion or wrinkling was noted with any treatment.

Raw product characterization

Final mix temperature was 0.7°C higher ($P < 0.05$) for high-fat than low-fat controls. Particle size did not consistently affect final mixing temperature, and preblending increased ($P < 0.05$) final mixing temperatures of treatments by 1°C. The mean pH of the N raw product was higher ($P < 0.05$) for the high-fat controls (6.42) than the low-fat controls (6.29). The pH tended to increase as FS addition increased. However, FS particle size and preblending did not consistently affect pH (data not shown).

Raw product pumpability was not different ($P > 0.05$) between either low- or high-fat, P or N controls and FS treatments. Preblending, FS particle size, and FS levels did not show consistent effects on raw product pumpability (data not shown). Eilert et al. (1991a) related rheological differences to the addition of flaked sinew in low-fat frankfurters. Raw batters with

Table 5—Sensory analysis of nonpreblended (N) and preblended (P) cooked salamis with or without large (L) and small (S) particle sizes of flaked sinew (FS) (means and standard error)

Treatments[hi]	Moisture	Shear force	Deformation	Firmness	Springiness	Cohesiveness
NControl 10%	7.5^{f}	12.0^{abc}	9.2^{cd}	6.8^{abcd}	7.4^{ab}	6.4^{b}
NControl 20%	8.2^{bcd}	11.3^{bcde}	10.1^{bcd}	6.6^{bcd}	7.2^{abc}	6.2^{bc}
NL 7.5% FS	8.5^{b}	11.9^{abc}	9.5^{cd}	6.4^{cd}	6.9^{bcde}	6.6^{b}
NL 15% FS	8.4^{bc}	11.8^{abc}	9.6^{cd}	6.9^{abc}	7.2^{abc}	6.4^{b}
NL 22.5% FS	7.9^{cdef}	12.1^{ab}	10.1^{bc}	6.6^{bcd}	6.9^{bcde}	6.4^{b}
NS 7.5% FS	8.3^{bc}	11.4^{bcde}	9.9^{bcd}	6.4^{cd}	6.5^{de}	6.3^{bc}
NS 15% FS	9.6^{a}	10.7^{e}	11.3^{a}	4.7^{f}	5.2^{f}	7.8^{a}
NS 22.5% FS	7.5^{f}	11.6^{abcd}	10.1^{bcd}	7.4^{a}	7.6^{a}	5.8^{c}
PControl 10%	8.0^{bcde}	11.2^{cde}	9.5^{cd}	6.1^{d}	6.9^{bcde}	6.4^{b}
PControl 20%	7.7^{def}	11.4^{bcde}	9.7^{cd}	7.1^{ab}	7.1^{abcd}	6.2^{bc}
PL 7.5% FS	7.6^{ef}	11.5^{abcd}	9.2^{d}	6.9^{abc}	7.2^{abc}	6.4^{b}
PL 15% FS	8.2^{bcd}	11.7^{abc}	9.7^{dd}	6.2^{d}	6.7^{cde}	6.6^{b}
PL 22.5% FS	8.0^{bcdef}	12.1^{ab}	9.6^{cd}	6.7^{bcd}	6.4^{e}	6.5^{b}
PS 7.5% FS	8.4^{bc}	12.2^{a}	9.3^{cd}	6.6^{bcd}	7.1^{abcd}	6.7^{b}
PS 15% FS	7.7^{def}	11.9^{abc}	9.5^{cd}	6.8^{abcd}	7.1^{abcd}	6.2^{bc}
PS 22.5% FS	9.3^{a}	10.8^{de}	10.8^{ab}	5.4^{e}	5.3^{f}	7.4^{a}
SE	0.2	0.3	0.3	0.2	0.2	0.2

[a-g] Means within the same column with same superscript letters do not differ (P> 0.05).
[h] Control treatments were formulated to contain 10% or 20% fat.
[i] The percent value indicated on all FS added treatments represents sinew content.

Table 6—Sensory analysis of nonpreblended (N) and preblended (P) cooked salamis with or without large (L) and small (S) particle sizes of flaked sinew (FS) (means and standard error)

Treatments[hi]	Mastication	Spice	Saltiness	FS residue	FS size	Astringent
NControl 10%	6.9^{ab}	9.1^{ab}	9.0^{abc}	8.1^{bcd}	7.4^{bcde}	4.6^{abc}
NControl 20%	6.4^{bcd}	8.8^{abc}	9.0^{abc}	8.2^{abc}	8.2^{a}	4.5^{abc}
NL 7.5% FS	6.5^{bcd}	9.2^{a}	9.1^{ab}	6.7^{f}	7.0^{de}	4.6^{ab}
NL 15% FS	6.6^{abcd}	8.4^{bc}	8.7^{abc}	7.0^{ef}	7.1^{cde}	4.1^{d}
NL 22.5% FS	6.5^{bcd}	8.9^{abc}	8.9^{abc}	7.6^{cde}	7.3^{bcde}	4.4^{abcd}
NS 7.5% FS	6.1^{d}	8.6^{abc}	8.7^{bc}	8.4^{ab}	7.8^{ab}	4.4^{abcd}
NS 15% FS	4.9^{e}	8.3^{c}	9.0^{abc}	8.0^{bcd}	7.5^{bcde}	4.4^{abcd}
NS 22.5% FS	7.2^{a}	8.8^{abc}	8.7^{c}	8.9^{a}	7.6^{abcd}	4.3^{cd}
PControl 10%	6.5^{bcd}	9.1^{ab}	9.1^{a}	8.3^{abc}	7.7^{abcd}	4.7^{a}
PControl 20%	6.7^{abc}	8.6^{abc}	8.7^{abc}	8.3^{abc}	7.8^{abc}	4.3^{abc}
PL 7.5% FS	6.7^{abc}	8.9^{abc}	8.9^{abc}	7.8^{bcd}	7.8^{abc}	4.6^{abc}
PL 15% FS	6.3^{cd}	9.2^{a}	9.0^{abc}	7.6^{cde}	7.1^{bcde}	4.5^{abc}
PL 22.5% FS	6.3^{cd}	9.0^{ab}	8.9^{abc}	7.5^{de}	7.3^{bcde}	4.5^{abc}
PS 7.5% FS	6.3^{cd}	9.2^{a}	9.0^{abc}	6.7^{f}	6.9^{e}	4.6^{abc}
PS 15% FS	6.7^{abcd}	9.0^{ab}	8.9^{abc}	8.2^{abcd}	7.8^{ab}	4.4^{abcd}
PS 22.5% FS	5.4^{e}	8.7^{abc}	9.1^{ab}	7.9^{bcd}	7.6^{abcde}	4.4^{abcd}
SE	0.2	0.3	0.1	0.3	0.3	0.1

[a-g] Means within the same column with same superscript letters do not differ (P>0.05).
[h] Control treatments were formulated to contain 10% or 20% fat.
[i] The percent value indicated on all FS added treatments represents sinew content.

high levels of connective tissue (30 and 40%) required more force for extrusion, further indicating their more viscous nature. Sadowska et al. (1980) attributed the increase in viscosity of meat batters to the interaction of denatured collagen with water.

Cooking yield, cooked pH, and purge loss

Relative to controls, yield decreased ($P < 0.05$) with addition of FS regardless of particle size (Table 2). Addition of 22.5% FS reduced yields by ≈4% compared to the 10% fat controls. This results were not in agreement with Quint (1987) who explored beef connective tissue addition (0, 10, 20 and 30%) to comminuted meat products. He noted that thermal processing yields increased with an increase in connective tissue and an increase in fat. Moreover, our yield data did not correspond with Gillet's (1987) results, as he observed no notable decreases in product yield with increase in collagen level.

Preblending did not consistently affect cooking yields. Perhaps the absence of any noticeable improvement in cooking yield indicated adequate myofibrillar protein extractability in both formulations (preblend and nopreblend) to bind available water and fat (Pepper and Schmidt, 1975; Maesso et al., 1970). Cooked product pH was not affected ($P > 0.05$) by FS level, FS particle size, or preblending.

Relative to controls, purge accumulation decreased ($P < 0.05$) in both slices and chubs of cooked salami with added FS of either S or L particle size. This may have been due to increased moisture loss during heat processing of the FS treatments. In addition, probably better protein-water interactions (Regenstein, 1984) occurred upon heating and then cooling of the FS treatments. When compared to N controls, preblending also reduced ($P < 0.05$) purge.

Color evaluations

The CIE values for Illuminant C (Table 3) showed that L* values increased (lighter color) ($P < 0.05$) for the FS treatments when compared to the N low-fat control. This confirmed results of Ambrosiadis and Wirth (1984), who reported that comminuted products extended with connective tissue were lighter and less red than controls. The P low-fat control was lighter than the N low-fat control. The CIE a* values were lowest ($P < 0.05$) for the high-fat controls when compared to the low-fat controls. The L*, a*, and b* values for Illuminant A (data not shown) followed the same patterns as those for Illuminant C, but were higher. Cured color development, measured by the ratio of %R650/%R570 nm, indicated that the low-fat controls had more ($P < 0.05$) cured color than the high-fat controls. Few significant differences in L*, a*, and b* values and cured color intensity were related to FS level and FS particle size. Blackmer (1992) demonstrated that addition of connective tissue after the second pass through the Baader Desinewing machine, and then modified by freezing/shattering resulted in lighter low-fat ground beef patties compared to controls.

Texture measurements

Preblending reduced hardness (Table 4) of the controls, especially for the high-fat control ($P < 0.05$). Contrary to the pattern shown for N products, FS addition increased ($P < 0.05$) hardness for the P treatments regardless of particle size. N samples tended to decrease in cohesiveness (Table 4) as FS increased up to 15%, but beyond that level, cohesiveness increased ($P < 0.05$). P samples, however, showed a mean decrease ($P > 0.05$) in cohesiveness as FS level increased regardless of particle size. Low-fat salami controls tended ($P > 0.05$) to be less springy (Table 4) than high-fat controls. Preblending made the low-fat controls more ($P < 0.05$) springy, and FS tended ($P > 0.05$) to decrease springiness of preblended treatments.

Tensile peak and break force as well as total energy and break force/cm^2 were not affected consistently by treatments (data not shown). Preblending increased ($P < 0.05$) peak forces for both low-fat (from 8.4 to 10.8 kgf) and high-fat (from 6.5 to 8.9 kgf) controls. Results for Lee-Kramer shear peak force (kgf) per wt and total energy (kgf*mm) per wt. confirmed the lack of differences ($P > 0.05$) observed for Instron tensile strength measurements of FS treatments (data not shown). Our results confirmed those of Quint (1987) who demonstrated that addition of frozen, flaked beef connective tissue had no effect on instrumental textural measures of low-fat frankfurters. However they contradicted Eilert et al. (1991b) findings that higher Kramer shear values were observed in frankfurters with increased beef connective tissue. N treatments with the L particle size of FS had higher ($P < 0.05$) peak force values (2.8 kgf/g) than those with the S particle size (2.5 kgf/g).

Sensory analyses

Sensory analysis for the cooked salami (Tables 5 and 6) showed no differences ($P > 0.05$) among treatments for sensory traits. Therefore, connective tissue levels up to 22.5% could be added to cooked salami with minimal texture deviations from low- and high-fat controls. Contrary to these results, Chavez et al. (1985) found that hamburger patties extended with up to 20% hide collagen (82.9% moisture content) increased in sensory moisture. Whiting et al. (1987) reported that, in restructured beef products, perception of connective tissue was not related directly to the total amount of connective tissue in the product. Our finding no difference in sensory FS residue among FS treatments was in agreement with Whiting et al. (1987). However, Berry et al. (1988) reported that excess gristle occurred with a higher level of collagen (1.6% total collagen).

CONCLUSIONS

TRIMMING AND REINCORPORATION of connective tissue to processed meat products is a feasible alternative for production of new low-fat meat products. Addition of flaked sinew up to 22.5% could be used as an attractive texture modifier ingredient to low-fat, coarsely ground, processed products. Although FS addition significantly reduced yield compared to controls, it reduced purge and enabled substitution of a lower cost raw material during formulation.

REFERENCES

AOAC, 1984. *Official Methods of Analysis*, 13th ed. Association of Official Analytical Chemists, Washington, DC.

Acton, J.C. and Saffle, R.L. 1969. Preblended and prerigor meat in sausage emulsions. Food Technol. 23(1): 93–97.

Ambrosiadis, I. and Wirth, F. 1984. Comminution of connective tissue and temperature pattern in the manufacture of frankfurter-type sausage. Fleischwirtschaft 64(8): 945–950.

Berry, B.W., Smith, J.J., Secrist, J.J., and Douglass, L.A. 1988. Consumer response to restructured beef steaks processed to have varying levels of connective tissue. J. Food Qual. 11(1): 15–25.

Blackmer, D.S. 1992. Characterization of low-fat ground beef patties made with non-meat ingredients. M.S. Thesis. University of Nebraska-Lincoln, Lincoln, NE.

Bourne, M. 1978. Texture profile analysis. Food Technol. 32(7): 62–72.

Chavez, J., Henrickson, R.L., and Rao, B.R. 1985. Collagen as a hamburger extender. J. Food Qual. 8(4): 265–272.

Claus, J.R., Hunt, M.C., Kastner, C.L., and Kropf, D.H. 1990. Low-fat, high-added water bologna: Effects of massaging, preblending and time of addition of water and fat on physical and sensory characteristics. J. Food Sci. 55(2): 338–341.

Cross, H.R., Berry, B.W., Nichols, J.E., Elder, R.S., and Quick, J.A. 1978. Effect of desinewing versus grinding on textural properties of beef. J. Food Sci. 43: 1507–1509.

Eilert, S.J., Blackmer, D.S., Mandigo, R.W., and Calkins, C.R. 1991a. Characterization and feasibility of meat emulsions manufactured with modified beef connective tissue. Paper no. 447, presented at 51st Annual Meeting of Inst. of Food Technologists, Dallas, TX, June 2–5.

Eilert, S.J., Blackmer, D.S., Mandigo, R.W., and Calkins, C.R. 1991b. Characteristics of low-fat frankfurters manufactured with modified beef connective tissue. Presented at 44th Reciprocal Meat Conference, Manhattan, KS, June 9–12. American Meat Science Association, Chicago, IL.

Erdman, A.M. and Watts, B.M. 1957. Spectrophotometric determination of the color change in cured meat. J. Agric. Food Chem. 5: 453.

Gillett, T.A. 1997. Collagen in Meat Emulsions. Ch. 12, In *Advances in Meat Research*, Vol. 4, *Collagen as a Food*. A.M. Pearson, T.R. Dutson and A.J. Bailey, (Ed.), p. 223–249. Van Nostrand Reinhold Company, New York.

Hand, L.W., Hollingsworth, C.A., Calkins, C.R., and Mandigo, R.W. 1987. Effects of preblending, reduced fat and salt levels on frankfurter characteristics. J. Food Sci. 52: 1149–1151.

Hunt, M.C., Acton, J.C., Benedict, R.C., Calkins, C.R., Cornforth, D.P., Jeremiah, L.E., Olson, D.G., Salm, C.P., Savell, J.W., and Shivas, S.D. 1992. Guidelines for meat color evaluation. Recip. Meat Conf., Kansas State University, Manhattan, KS.

Hunt, M.C. and Campbell, R. 1992. Lean, fat, and connective tissue from beef shanks processed with a Baader desinewer. Dept. Animal Science and Industry, Kansas State University, Manhattan, KS.

Jones, K.W., Snyder, W.D., Mandigo, R.W., and Calkins, C.R. 1982a. Rheological and textural properties of meat emulsions with collagen. Amer. Soc. Animal Sci. Abstract No. 308. Guelph, Canada.

Jones, K.W., Snyder, W.D., Mandigo, R.W., and Calkins, C.R. 1982b. Effects of varying collagen levels on the emulsion properties of sausage batters. Amer. Soc. Animal Sci. Abstract No. 307, Guelph, Canada.

Kenney, P.B., Kastner, C.L., and Kropf, D.H. 1992. Raw and pre-heated epimysium and gelatin affect properties of low-salt, low-fat, restructured beef. J. Food Sci. 3: 551–554.

Liu, C.W., Huffman, D.L., Egbert, W.R., and Liu, M.N. 1990. Effects of trimming and added connective tissue on compositional, physical and sensory properties of restructured, pre-cooked beef steaks. J. Food Sci. 55: 1258–1263.

Maesso, E.R., Baker, R.C., and Vadehra, D.V. 1970. Effect of some physical and chemical treatments on the binding quality of poultry loaves. J. Food Sci. 35(4): 440–443.

Mandigo, R.W. and Eilert, S.J. 1993. Processing of meat to meet consumer demand: Developments in restructured and low-fat processed products. 39th International Congress of Meat Science & Technology. Calgary, Alberta, Canada.

Montejano, J.G., Hamann, D., and Lanier, T. 1985. Comparison of two instrumental methods with sensory texture of proteins gels. J. Text. Stud. 16(4): 403–424.

Ockerman, H.W. and Crespo, F.L. 1982. Physicochemical changes occurring during storage of precooked beef blends at different temperatures and two levels of salt. J. Food Sci. 47: 849–857.

Pepper, F.H. and Schmidt, G.R. 1975. Effect of blending time, salt, phosphate, and hot-boned beef on binding strength and cook yield of beef rolls. J. Food Sci. 40(2): 227–230.

Quint, L.N. 1987. Interaction of fat and connective tissue in meat emulsions. M.S. thesis, Univ. of Nebraska, Lincoln, NE.

Regenstein, J.M. 1984. Protein-water interactions in muscle foods. *Reciprocal Meat Conference Proceedings* 37: 44–51.

Sadowska, M., Sikorski, Z.E., and Dobosz, M. 1980. The influence of collagen on the rheological properties of meat homogenates. Lebensm.-Wiss.U.-Technol. 13(5): 232–236.

Saffle, R.L., Carpenter, J.A., and Moore, D.G. 1964. Peeling ease of frankfurters. 1. Effect of chemical composition, heat, collagen and type of fat. Food Technol. 18: 130.

SAS Institute, Inc., 1992. *SAS/STAT Users Guide*. SAS Institute Inc., Cary, NC.

Whiting, R.C., Strange, E.D., Cronlund, A.C., and Miller, A.J. 1987. Effect of connective tissue on the sensory textural scores and shear forces of restructured beef steaks. *33rd Int. Cong. Meat Sci. Technol.* 2(6:23): 320–322.

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Dietary Supplementation of Vitamin E to Feedlot Cattle Affects Beef Retail Display Properties

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ABSTRACT

Beef products from carcasses (n = 20) of cattle fed supplemental vitamin E and beef products from commodity carcasses (n = 20), used as controls, were compared to determine effects of increased vitamin E tissue levels on display appearance of fresh beef held in supermarket and simulated retail conditions. Meat from cattle fed supplemental vitamin E contained higher levels of α-tocopherol ($P < 0.05$) and, over time, exhibited less lipid oxidation, brighter lean color and lower discoloration. Monitoring supermarkets for discounted retail meats showed increased levels of α-tocopherol in beef extended caselife and decreased the incidence of discounted beef products.

Key Words: beef, vitamin E, color, retail display, feed supplement

INTRODUCTION

COLOR is an extremely critical component of appearance of fresh red meat and has substantial influence on purchase decisions. Kropf (1980) reported that consumers have learned through experience that the desirable color of fresh beef was bright cherry-red and that any deviation from this created a degree of unacceptability. Such perceived freshness primarily determines the retail caselife, or the length of time the product displays the desirable bright-red color. Extending the period of time that the color is bright-red should improve retail saleability.

Discoloration in retail meats during display conditions is a combined function of muscle pigment oxidation (oxymyoglobin to metmyoglobin) and lipid oxidation occurring in intramuscular fat, intermuscular fat and (or) membrane phospholipids. These two types of oxidation are closely related and have been hypothesized to be caused by similar processes (Faustman and Cassens, 1989). Extending the time beef retains its bright-red color can be accomplished by preventing or delaying pigment oxidation (minimizing formation of metmyoglobin). The state of the whole-muscle meat pigment is influenced by temperature, light source, microbial growth (Satterlee and Hansmeyer, 1974) and the oxygen permeability of the packaging film (Kropf, 1980).

Vitamin E functions as a lipid-soluble antioxidant in cell membranes (Linder, 1985), and its most active form is α-tocopherol (Tannenbaum et al., 1985). Previous research has shown the beneficial effects of dietary vitamin E supplementation on meat products obtained from carcasses of swine (Yamauchi et al., 1980), poultry (Bartov and Bornstein, 1977) and cattle (Faustman et al., 1989; Arnold et al., 1993).

The objective of our study was to determine the effects of increased α-tocopherol levels in beef on storage quality of beef retail meats during actual and simulated retail display.

MATERIALS & METHODS

CROSSBRED STEERS (n = 80) from a single source (Ainsworth Feedyard, Ainsworth, Nebraska) were used. The steers were fed a conventional finishing diet which was supplemented with vitamin E in the form of dl-α-tocopheryl acetate (Hoffmann LaRoche, Inc., Nutley, NJ) throughout the 123-day feeding period at a level of 500 IU/animal/day. Beef from these steers will be referred to as VITE.

The steers were transported to a beef packing plant where they were humanely slaughtered using conventional commercial procedures. Following a 24 hr chill (0 ± 2°C), carcasses were evaluated and USDA Quality Grades were recorded. Twenty VITE carcasses which graded USDA Choice were randomly sampled from the Ainsworth source and, 20 commodity USDA Choice carcasses were selected randomly from the inventory of the beef packing plant and used as controls (CONT). Guidelines of the Institutional Meat Purchase Specifications (IMPS) (NAMP, 1988) were followed during fabrication of the short loin (IMPS #174), round knuckle (IMPS #167) and chuck roll (IMPS #116A) subprimal cuts. During fabrication, a 10-g sample of muscle (chuck roll = *Longissimus* muscle, short loin = *Longissimus* muscle, and round knuckle = *Vastus intermedius, Vastus lateralis, and/or Rectus femoris*) was randomly obtained from eight of the VITE subprimals and eight of the CONT subprimals for α-tocopherol analysis. Samples were transported to Colorado State University where they were packaged in aluminum foil and frozen (−70°C) until subsequent analyses. The level of α-tocopherol was determined in duplicate muscle samples, following saponification and extraction (Craig, 1994), by reverse-phase high performance liquid chromatography and fluorescence detection (Craig et al., 1989).

Following vacuum packaging, the short loins and round knuckles were transported via refrigerated truck to a central distribution warehouse in Denver, CO. The bulk combo-packed chuck rolls were delivered to a beef grinding plant and were ground through a plate with 1.27 cm orifices, packaged in 4.54 kg chub casing packages and transported to the same central distribution warehouse. All samples were stored until 12 days postmortem at 1–2°C in the same central distribution warehouse. Following storage, the VITE and CONT short loins, round knuckles and ground chuck chubs were transported to two retail outlets in Fort Collins, CO. Upon arrival, the short loins and round knuckles were fabricated into T-bone (IMPS #1174) and round knuckle (IMPS #1167) steaks, respectively, as needed to stock retail cases. The coarse-ground chuck was ground a second time through a plate with 0.32 cm orifices and divided into various weight-portions for retail sale. All retail products were placed on polyfoam trays, overwrapped in oxygen-permeable (1000–1050 mL O_2/645 cm^2/24 hr), polyvinylchloride packaging film and placed in a commercial retail display case (1–2°C) under cool-white fluorescent lighting (350–750 lux). A small distinguishing mark was placed on each retail package to differentiate the two treatments. About four times daily, University personnel counted the retail packages to determine the number that had been sold, discounted in price and/or discarded. Retail packages discounted in price and/or discarded were quantified by the meat market managers at both cooperating stores, with the assistance of University personnel.

A subsample of the retail meats (T-bone steaks, n = 3; round knuckle steaks, n = 3; and ground chuck, n = 3) was obtained from each of the retail stores and transported to the Colorado State University Meat Laboratory. Samples were displayed under cool-white fluorescent lighting (350–750 lux) in a refrigerated (1–2°C) retail-simulation area for a 7-day period. On each day of display, the retail meats were visually evaluated twice (7:00 am and 5:00 pm) by a four-member trained panel for lean color and percent discoloration (Table 1). Scores from the daily evaluations from all evaluators were averaged prior to statistical analysis. On display days 1 and 7, a 10-g muscle sample (ground chuck, T-bone steak = *Longissimus* muscle, round knuckle steak = *Vastus intermedius, Vastus lateralis*, and(or) *Rectus femoris*) was obtained from each of the retail items to evaluate the extent of lipid oxidation. This was measured using the thiobarbituric acid (TBA) test procedure of Salih et al. (1987), with the modification of 5% (w/v) aqueous trichloroacetic acid (Mallincrodt, Inc., Paris, KY) used for extraction solvent. Reported values for TBA number were expressed as mg malonaldehyde/kg muscle.

Data were analyzed as a completely random design for each day of display except for visual assessment data (lean color and percent dis-

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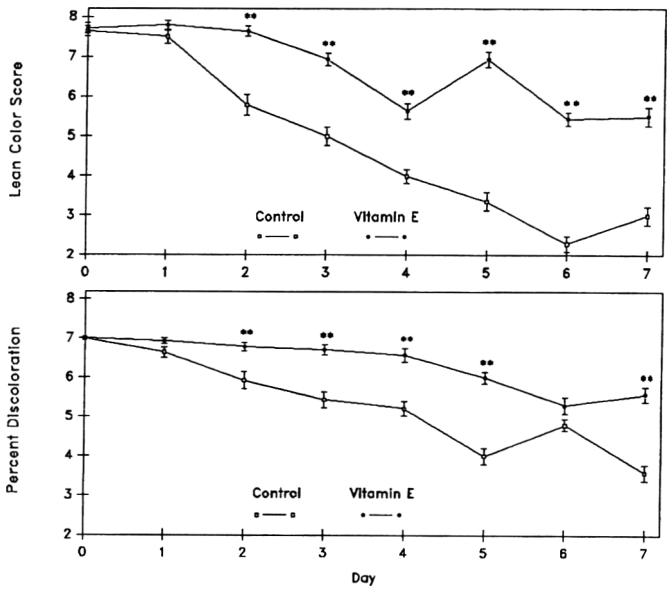

Fig. 1—Changes in visual assessment scores for T-bone steaks from CONT and VITE with time of display. Values represent means ± SEM. * = denotes significance ($P < 0.05$). ** = denotes significance ($P < 0.01$).

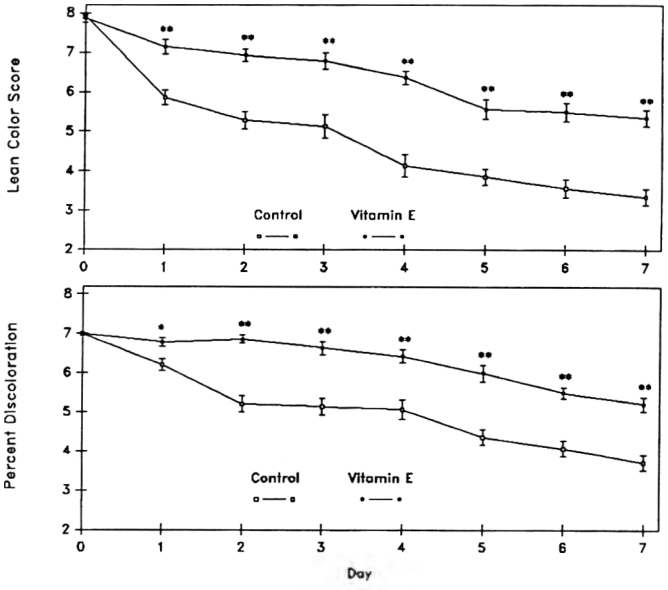

Fig. 2—Changes in visual assessment scores for round knuckle steaks from CONT and VITE with time of display. Values represent means ± SEM. * = denotes significance ($P < 0.05$). ** = denotes significance ($P < 0.01$).

Table 1—Visual evaluation scales for lean color and percent discoloration

Lean color (oxygenated)	Percent surface discoloration
8—Bright cherry-red	7—None
7—Moderately bright cherry-red	6—1–10
6—Cherry-red	5—11–25
5—Slightly dark-red	4—26–50
4—Moderately dark-red or brown	3—51–75
3—Dark-red or brown	2—76–99
2—Very dark-brown	1—Complete
1—Extremely dark-brown or green	

Table 2—Least squares means for α-tocopherol level and TBA number

	Short loin		Round knuckle		Chuck roll	
	VITE	CONT	VITE	CONT	VITE	CONT
α-Tocopherol (μg/g muscle)	4.7*	3.5	6.9**	3.4	5.2**	2.8
TBA number[a]						
day 1	0.18	0.19	0.19*	0.26	0.35**	0.18
day 7	0.31**	0.94	1.11**	2.00	0.42**	0.90

[a] TBA number=mg malonaldehyde/kg muscle.
*Denotes differences between VITE and CONT at $P<0.05$.
**Denotes differences between VITE and CONT at $P<0.01$.

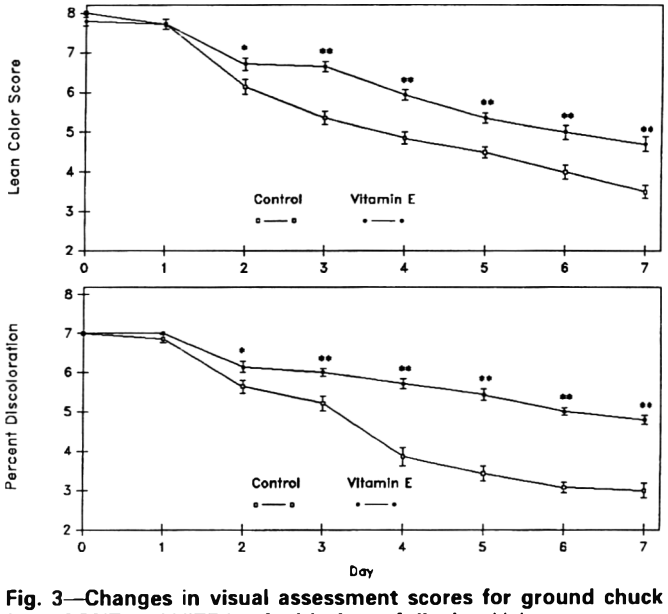

Fig. 3—Changes in visual assessment scores for ground chuck from CONT and VITE beef with time of display. Values represent means ± SEM. * = denotes significance ($P < 0.05$). ** = denotes significance ($P < 0.01$).

coloration) which were analyzed as repeated measures (Freund et al., 1986).

RESULTS & DISCUSSION

THE α-TOCOPHEROL CONCENTRATIONS for each beef subprimal were higher for VITE than CONT (Table 1). Results, compared by subprimal cut, indicated that amounts of α-tocopherol were greater in VITE by 34%, 84% and 103% for short loin, chuck roll and round knuckle, respectively. This elevation was expected because supplemental feeding of vitamin E has been shown to increase tissue content of α-tocopherol (Faustman et al., 1989).

Simulated retail visual assessments of lean color and percent discoloration of CONT and VITE meats (Fig. 1, 2, and 3) showed lean color became less desirable as display time increased, and percent discoloration increased. No differences ($P > 0.01$) in lean color and percent discoloration between CONT and VITE T-bone steaks were detected on days 0 and 1 (Fig. 1). VITE T-bone steaks had desirable lean color throughout retail display ($P < 0.01$) whereas CONT T-bone steaks developed dark red or brown colors by the end of the period. VITE T-bone steaks had less discoloration ($P < 0.01$) than CONT T-bone steaks on days 2, 3, 4, 5 and 7. VITE round knuckle steaks were brighter than CONT ($P < 0.01$) in lean color (Fig. 2) and had less discoloration starting on day 1 and continuing throughout day 7. VITE round knuckle steaks remained cherry-red in lean color, with < 25% surface discoloration, throughout the display period whereas CONT round knuckle steaks developed a dark red or brown lean color and more discoloration. Ground chuck (Fig. 3) had bright lean color and very little discoloration on days 0 and 1 for both VITE and CONT ground chuck. Lean color and percent discoloration on days 2–7 for CONT ground chuck decreased more rapidly than did that for VITE ground chuck. Arnold et al. (1993) found that feeding supplemental

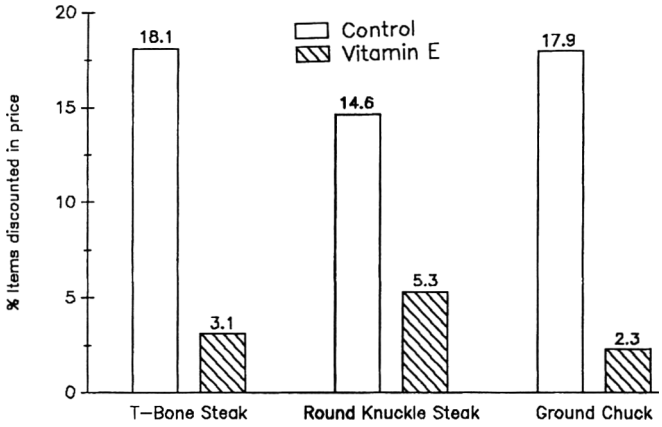

Fig. 4—Effects of dietary vitamin E supplementation on percentage of retail meats discounted when displayed under supermarket conditions.

vitamin E to cattle extended the days (by 2 to 5 days) beef could be displayed until unacceptable.

Analyses for thiobarbituric acid (TBA), on days 1 and 7 of retail display (Table 2) were used to quantitate lipid oxidation. On day 7, VITE meats had less lipid oxidation than CONT by 33 to 50% of that ($P < 0.01$) in CONT retail items. These results confirmed those of Faustman et al. (1989) who found that beef from Holstein steers fed supplemental vitamin E was less susceptible to pigment and lipid oxidation. Retail supermarket display data revealed that VITE decreased the number of discounted retail meats (Fig. 4) when compared to CONT retail meats.

CONCLUSION

SUPPLEMENTAL FEEDING OF VITAMIN E to feedlot cattle can greatly improve caselife of fresh beef retail products. The lean color and percent discoloration of VITE compared to CONT was improved due to decrease in oxidation of pigment and lipids. Using supermarket and simulated retail-display conditions, the VITE retail items remained acceptable for a longer period of time than did CONT retail items. These advantages will improve the profitability of beef in the retail case thereby improving the competitiveness of beef as a protein source.

REFERENCES

Arnold, R.N., Scheller, K.K., Arp, S.C., Williams, S.N., and Schaefer, D.M. 1993. Dietary α-tocopherol acetate enhances beef quality in Holstein and beef breed steers. J. Food Sci. 58: 28–33.

Bartov, I. and Bornstein, S. 1977. Stability of abdominal fat and meat of broilers: The interrelationships between the effects of dietary fat and vitamin E supplements. Brit. Poult. Sci. 18: 47–57.

Craig, A.M. 1994. Personal communication. Oregon State University, Corvallis, OR.

Craig, A.M., Blythe, L.L., Lassen, E.D., Rowe, K.E., Barrington, R., and Slizeski, M. 1989. Variations of serum vitamin E, cholesterol, and total serum lipid concentrations in horses during a 72-hour period. Am. J. Vet. Res. 50: 1527–1531.

Faustman, C. and Cassens, R.G. 1989. Strategies for improving fresh meat color. Proc. 35th International Cong. of Meat Sci. and Technol., August 20–25, 1989, Copenhagen, Denmark.

Faustman, C., Cassens, R.G., Schaefer, D.M., Buege, D.R., Williams, S.N., and Scheller, K.K. 1989. Improvement of pigment and lipid stability in Holstein steer beef by dietary supplementation with vitamin E. J. Food Sci. 54: 858–862.

Freund, R.J., Littell, R.C., and Spector, P.C. 1986. *SAS System for Linear Models*. SAS Institute, Inc., Cary, NC.

Kropf, D.H. 1980. Effects of retail display conditions on meat color. Proc. Reciprocal Meat Conf., June 22–25, 1980, Purdue University. 33: 15–32.

Linder, M.C. 1985. Nutrition and metabolism of vitamins. In *Nutritional Biochemistry and Metabolism*, M.C. Linder (Ed.), New York.

NAMP. 1988. *The Meat Buyer's Guide*. National Association of Meat Purveyors, Reston, VA.

Salih, A.M., Smith, D.N., Price, J.F., and Dawson, L.E. 1987. Modified extraction 2-thiobarbituric acid method for measuring lipid oxidation in poultry. Poultry Sci. 66: 1483–1488.

Satterlee, L.D. and Hansmeyer, W. 1974. The role of light and surface bacteria in the color stability of prepackaged beef. J. Food. Sci. 39: 305–308.

Tannenbaum, S.R., Young, V.R., and Archer, M.C. 1985. Vitamins and minerals. Ch. 7, In *Food Chemistry*, O.R. Fennema (Ed.), p. 476–544. Marcel Dekkar, Inc., New York.

Yamauchi, K., Nagai, Y., and Ohashi, T. 1980. Quantitative relationship between alpha-tocopherol and polyunsaturated fatty acids and its connection to development of oxidative rancidity in porcine skeletal muscle. Agric. Biol. Chem. 44: 1061–1067.

Ms received 2/23/94; revised 10/21/94; accepted 11/4/94.

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Lactate Dehydrogenase Monoclonal Antibody Immunoassay for Detection of Turkey Meat in Beef and Pork

CHENG-HSIN WANG and DENISE M. SMITH

ABSTRACT

Relative epitope binding positions on turkey muscle LDH of four monoclonal antibodies (MAbs) was determined by comparing the absorbance by sandwich ELISA from different combinations of capture and biotin-labeled detector MAbs. Epitopes for MAbs E6B and B3C were close to each other. Binding of MAb B3C and G4D did not inhibit binding of D5E to LDH, thus a sandwich ELISA using D5E as capture MAb and biotin-labeled B3C as detector MAb was developed. The LDH MAb sandwich ELISA detected 1% adulteration of turkey breast or thigh muscle in raw beef and pork.

Key Words: ELISA, lactate dehydrogenase, turkey, adulteration

INTRODUCTION

IMMUNOASSAYS are used to detect adulteration, additives, microorganisms, mycotoxins, pesticides, drugs, and other constituents in foods (Rittenburg, 1990; Samarajeewa et al., 1991; Morgan et al., 1992). Enzyme-linked immunosorbent assays (ELISAs) with monoclonal antibodies (MAbs) have been developed to study changes in structure and epitope (antigenic determinant) regions of proteins, such as ovalbumin (Varshney et al., 1991; Ikura et al., 1992) and β-lactoglobulin (Kaminogawa et al., 1989) during heat treatment.

Epitope mapping has been used to determine whether individual MAbs developed against the same antigen bind to identical or overlapping epitopes (Harlow and Lane, 1988). When epitopes are located in relatively close proximity on the antigen's surface, binding for MAbs will be mutually competitive (Tzartos et al., 1981; Kordossi and Tzartos, 1987). Thus those two MAbs could not be used as the pair of capture and detector MAbs in sandwich ELISA. Therefore, epitope mapping is required to develop MAb sandwich ELISA. Competitive ELISAs have been used to investigate the relative proximity of epitopes recognized by different MAbs (Smith-Gill et al., 1984; Pfeiffer et al., 1987; Smith and Wilson, 1992). For example, Smith and Wilson (1992) found that 23 MAbs against rat brain hexokinase were divided into 9 groups based on binding to defined surface regions of the N- or C-terminal domains of the enzyme using competitive epitope mapping methods.

Detecting adulteration of meat products with unlabeled meat species is important for economic, health and religious reasons (Andrews et al., 1992). Several ELISAs have been developed for detection of species adulteration in raw meat using polyclonal antibodies (PAb) against serum albumins (Jones and Patterson, 1986; Ayob et al., 1989) or sarcoplasmic proteins (Martin et al., 1988a, b). In those assays it was necessary to reduce species cross-reactivity by immunosorbent affinity chromatography of each batch of polyclonal antibodies. To eliminate this problem and insure a continuous and consistent supply of antibody, several monoclonal-based ELISAs were developed. Martin et al. (1991) described the preparation of sandwich ELISA for the detection of chicken meat. Monoclonal antibodies specific for a chicken muscle soluble protein, tentatively identified as 3-phosphoglycerate kinase (Martin et al., 1989), was used as the capture antibody (Martin et al., 1991). Polyclonal antibodies were used as detector antibodies.

Polyclonal antibodies against chicken muscle lactate dehydrogenase (LDH) and turkey muscle LDH, and four MAbs against chicken muscle LDH have been produced in our laboratory (Wang et al., 1992; Abouzied et al., 1993). Chicken muscle LDH MAbs cross-reacted with turkey muscle LDH, but not with LDH from other species (Abouzied et al., 1993). Because of the species specificity of LDH MAbs and unlimited supply of MAbs, it would be highly advantageous to develop an LDH sandwich ELISA using only monoclonal antibodies to detect meat adulteration. Our objective was to determine the relative position of MAb epitopes on turkey muscle LDH to design an effective monoclonal-based sandwich ELISA, and to investigate the feasibility of using this ELISA to detect turkey muscle in beef and pork products.

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MATERIALS & METHODS

Materials

Polyoxyethylenesorbitan monolaurate (Tween 20), 2,2'-azino-bis (3-ethylbenzthiazoline-6-sulfonic acid) (ABTS), biotinamido-caproate N-hydroxysuccinimide ester, dimethyl sulfoxide and avidin-horseradish peroxidase conjugate were purchased from Sigma Chemical Co. (St. Louis, MO). Bovine serum albumin (BSA) (Fraction V) was from Amresco (Solon, OH). Microtiter plates (Immunolon-2 Removawells) were from Dynatech Laboratories (Alexandria, VA). Turkey breast muscle LDH was purified using ammonium sulfate and acetone precipitation (Wang et al., 1992). All other chemicals were of reagent grade or better.

Conjugation of biotin to LDH MAbs

Production of MAbs against chicken muscle LDH was described previously (Abouzied et al., 1993). Four MAbs (D5E, B3C, G4D and E6B) were purified with 50% ammonium sulfate precipitation (Abouzied et al., 1993) and a protein G affinity column (Pierce, Rockford, IL). Biotin was conjugated to purified MAbs according to Harlow and Lane (1988).

Titration for biotin-labeled MAbs

Biotin-labeled MAbs were titrated to determine the appropriate concentration to use in sandwich ELISA. Microtiter wells were coated overnight (4°C) with 100 µL turkey breast muscle LDH (5 µg/mL) in 0.1M sodium borate buffer (pH 9.6). Plates were washed four times with 0.15M NaCl, 0.01M Na phosphate buffer, pH 7.2, containing 0.05% (v/v) Tween 20 (PBS-Tween). Wells were incubated with 300 µL 1% BSA (w/v) in 0.15M NaCl, 0.1 M Na phosphate buffer, pH 7.2, containing 0.2% (v/v) Tween 20 (BSA-PBST) at 37°C for 30 min to minimize nonspecific binding. After washing three times with PBS-Tween, 50 µL biotin-labeled MAb diluted (1:50–1:800) in BSA-PBST was added to each well. After incubation for 1 hr at 37°C and washing five times with PBS-Tween, 100 µL avidin peroxidase conjugate (1/1500 in BSA-PBST) was added to each well and incubated for 30 min at 37°C. Plates were then washed 8 times with PBS-Tween. Bound peroxidase activity was determined by adding 100 µL substrate solution (0.1M citrate buffer, pH 4.0, containing 0.025% (w/v) ABTS and 0.02% (v/v) H_2O_2) to each well. After 10 min at 25°C, the reaction was stopped by adding 100 µL 0.3M citric acid containing 0.1% (w/v) Na azide to each well. Absorbance was read at 405 nm using a Minireader II (Dynatech).

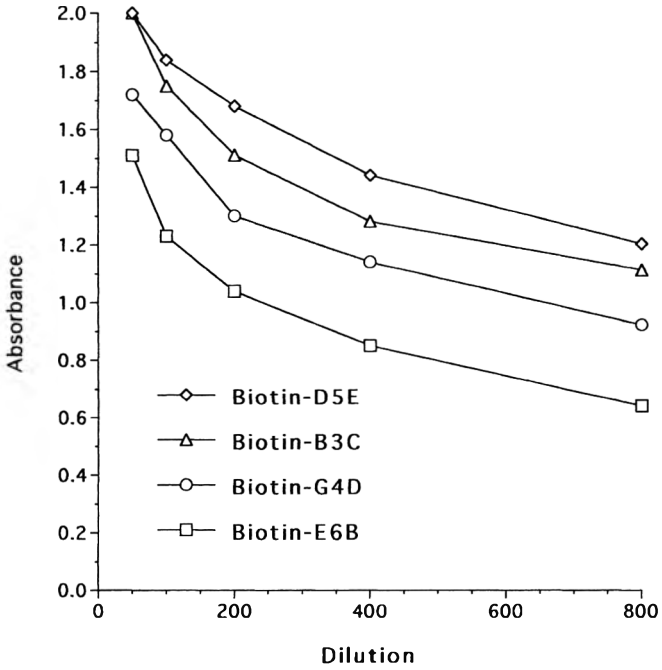

Fig. 1—Titration results for biotin-labeled lactate dehydrogenase (LDH) monoclonal antibodies (MAb). Microtiter wells were coated with turkey muscle LDH. Serial dilutions of biotin-labeled LDH MAbs were used (avg of three replicate values).

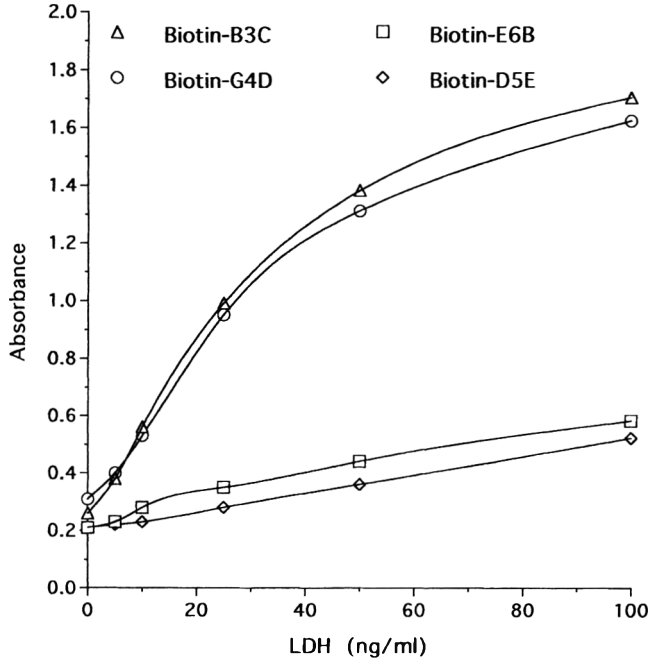

Fig. 2—Sandwich enzyme-linked immunosorbent assay for epitope mapping of lactate dehydrogenase (LDH) monoclonal antibodies (MAb) relation to level of LDH. Microtiter wells were coated with MAb D5E as capture antibody. Biotin-labeled D5E, B3C, G4D and E6B at 400, 200, 125 and 50-fold dilution, respectively, were used as detector antibodies (avg of three replicate values).

Sandwich ELISA for epitope mapping

Microtiter wells were coated with 100 µL MAb (as capture antibodies) diluted (1/400 for D5E, 1/100 for B3C and G4D, and 1/50 for E6B) in 0.1M carbonate buffer (pH 9.6) and dried overnight at 40°C in a forced-air oven. Wells were washed four times with PBS-Tween, and 300 µL BSA-PBST was added to each well. Incubation was 30 min at 37°C. After washing three times with PBS-Tween, 100 µL turkey breast muscle LDH in BSA-PBST was added and incubated at 37°C for 1 hr. Plates were washed four more times with PBS-Tween, and 100 µL biotin-labeled MAb (as detector antibodies) diluted in BSA-PBST was added. After incubation for 1 hr at 37°C and washing four times with PBS-Tween, 100 µL avidin peroxidase conjugate diluted (1:1500) in BSA-PBST was added to each well. Plates were incubated for 30 min at 37°C and washed eight times with PBS-Tween. Bound peroxidase activity and absorbance was determined as described. Relative position of epitopes was determined by comparing absorbance of sandwich ELISA from different combinations of capture antibodies and detector antibodies.

Detection of turkey in meat

Pork sirloin, lean ground beef, turkey thigh and skinless ground turkey breast were purchased from a local market. Pork or skinless turkey thigh was ground through the 4-mm plate of a KitchenAid grinder (Hobart Corp., Troy, OH). Meat (50g) containing 1, 3, 10 and 20% (w/w) ground turkey breast or thigh was homogenized with 150 mL 0.15M NaCl, 0.01M Na phosphate buffer (pH 7.2) in a Waring Blendor for 90 sec. The homogenate was centrifuged at 16000 × *g* for 20 min at 4°C, and the supernatant was filtered through Whatman No. 1 filter paper. Meat extracts were diluted (1:100–1:10000) in BSA-PBST and the amount of turkey muscle LDH was determined by sandwich ELISA. Purified turkey breast muscle LDH (0–100 ng/mL) was used to prepare a standard curve in each plate, and results were expressed as µg LDH/g meat.

Statistics

Simple linear regression analysis was performed with a Lotus 1-2-3 program (Version 3.1, Lotus Development Corporation, Cambridge, MA). All experiments were performed in triplicate.

RESULTS & DISCUSSION

Epitope mapping

For epitope mapping, each biotin-labeled detector MAb must be used at a dilution that produces equal absorbance in ELISA. The concentrations of biotin-labeled MAbs we used were based on titration curves (Fig. 1). A similar ELISA absorbance (about 1.5) was observed for biotin-labeled D5E, B3C, G4D and E6B at 400, 200, 125 and 50-fold dilution, respectively. Thus, these dilutions of biotin-labeled MAbs were used in our study for epitope mapping.

When D5E was used as capture antibody, ELISA absorbance for biotin-labeled B3C and G4D increased from about 0.3 to 1.6–1.7 as LDH concentration was increased to 100 ng/mL (Fig. 2). These results suggested that the epitopes for B3C and G4D were separated from D5E such that binding of LDH to capture MAb (D5E) did not inhibit the binding of detector MAbs (biotin-labeled B3C or G4D) to LDH. Therefore, peroxidase-conjugated avidin could bind biotin-labeled MAb and color development was observed.

The ELISA absorbance for biotin-labeled E6B increased from about 0.2 to 0.55 as LDH concentration was increased to 100 ng/mL when D5E was used as capture antibody (Fig. 2). This suggested that epitopes for E6B and D5E were close to each other, and binding of D5E to LDH inhibited access for biotin-labeled E6B. Since both unlabeled and labeled D5E recognized the same epitope, ELISA absorbance for biotin-labeled D5E as detector antibody was very low (about 0.5 at 100 ng/mL LDH) as expected. An average protein epitope reportedly covers an area of about 2–7 nm^2, and the antigen binding region of immunoglobulin G binds to a circular area of about 10 nm^2 with a diameter of about 3.5 nm on the surface of the antigen (Tzartos et al., 1981; Kordossi and Tzartos, 1987). Hence, the distance between epitopes for E6B and D5E is probably <3.5 nm; the binding sites for B3C and G4D are probably separated from D5E by a distance >3.5 nm. Although infrequent, binding of one antibody might possibly induce conformational changes that inhibit binding of the second antibody (Kordossi and Tzartos, 1987; Wilson, 1991).

The ELISA absorbance measured at 100 ng/mL LDH was about 0.70 for the pairs B3C and labeled B3C, and G4D and

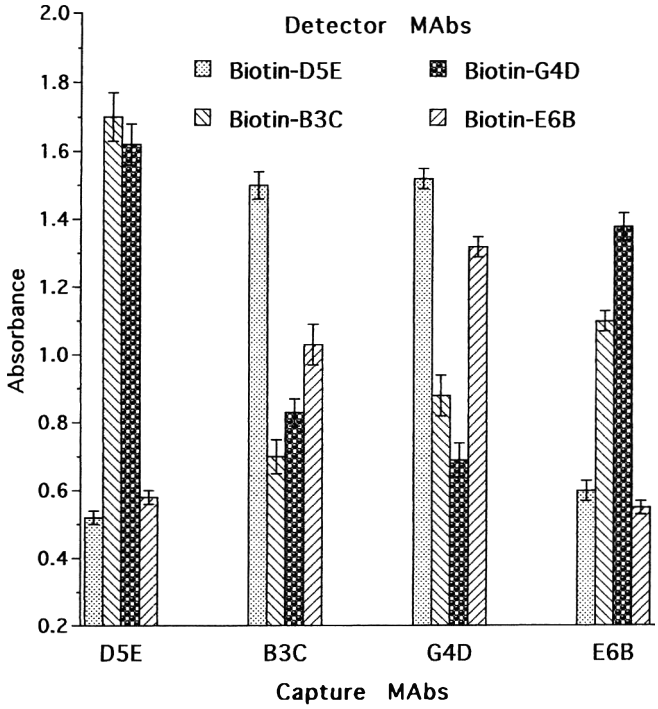

Fig. 3—Sandwich enzyme-linked immunosorbent assay (ELISA) for epitope mapping of lactate dehydrogenase (LDH) on different capture monoclonal antibodies (MAb). Microtiter wells were coated with different MAb as capture antibodies. Biotin-labeled D5E, B3C, G4D and E6B at 400, 200, 125 and 50-fold dilution, respectively, were used as detection antibodies. ELISA absorbance was measured at 100 ng/mL of turkey muscle LDH. Means ± standard deviation of three replicates are shown.

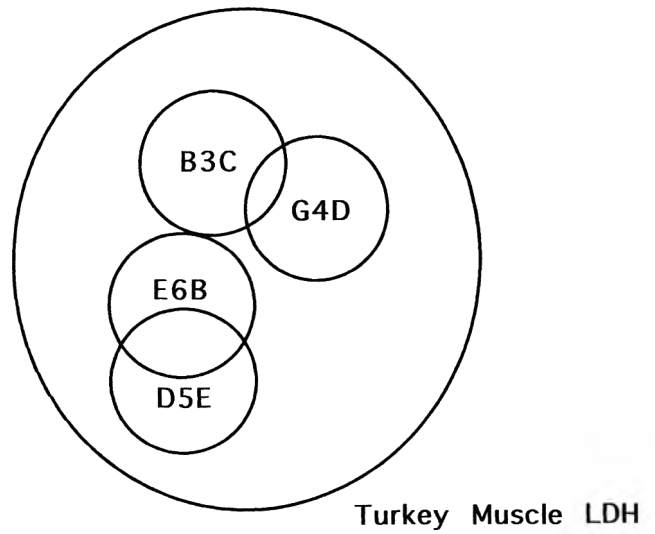

Fig. 4—Schematic diagram indicating relative position of epitopes for lactate dehydrogenase (LDH) monoclonal antibodies (MAb) deduced from sandwich enzyme-linked immunosorbent assay. Circles represent the regions of MAb epitopes on turkey muscle LDH. Overlapping circles suggest that binding of one MAb precludes binding of second MAb.

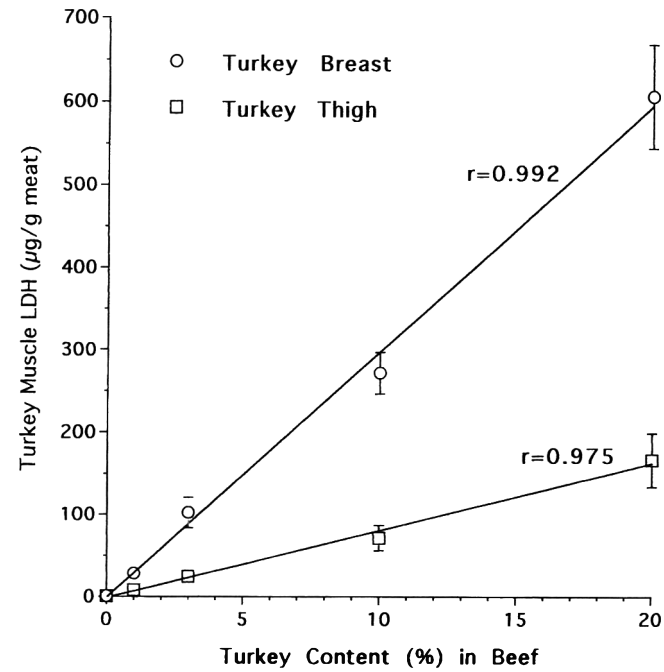

Fig. 5—Detection of turkey muscle lactate dehydrogenase (LDH) in beef containing different concentrations of turkey breast or thigh muscle using sandwich enzyme-linked immunosorbent assay. Microtiter wells were coated with monoclonal antibody D5E (400-fold dilution) as capture antibody. Biotin-labeled B3C (200-fold dilution) was used as detector antibody. Bars indicate standard deviation of the mean for three replicate values. Correlation coefficient (r) was determined by linear regression.

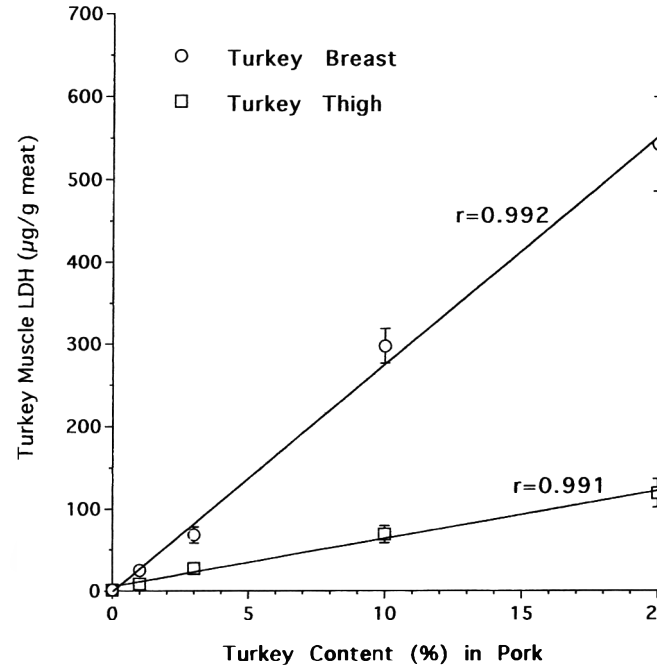

Fig. 6—Detection of turkey muscle lactate dehydrogenase (LDH) in pork containing different concentrations of turkey breast or thigh muscle using sandwich enzyme-linked immunosorbent assay. Microtiter wells were coated with monoclonal antibody D5E (400-fold dilution) as capture antibody. Biotin-labeled B3C (200-fold dilution) was used as detector antibody. Bars indicate standard deviation of the mean for three replicate values. Correlation coefficient (r) was determined by linear regression.

labeled G4D (Fig. 3). The absorbance was about 0.5–0.6 for the pairs D5E and labeled D5E, and E6B and labeled E6B at 100 ng/mL of LDH. The background absorbance (without LDH) was about 0.2–0.3. These results indicated that when the capture MAb was bound to turkey breast muscle LDH, binding sites were available for the same biotin-labeled detector MAb. We previously demonstrated that turkey breast muscle LDH contained 4 M subunits (Wang et al., 1992). Possibly the same epitopes were found on different M subunits of turkey breast muscle LDH, and capture antibodies did not bind to all the ep-

itopes on the surface of LDH. Hence, some epitopes were still available for biotin-labeled MAbs after LDH molecules were bound by the same capture MAbs.

When B3C was used as capture MAb, the difference in ELISA absorbance between biotin-labeled G4D and biotin-labeled B3C was about 0.1–0.2 (Fig. 3). This suggested that the epitopes for B3C and G4D were close to each other and the distance between these two epitopes was <3.5 nm. A similar result was also obtained using G4D as capture MAb. High ELISA absorbance (>1.30) was observed for the pairs G4D and biotin-labeled E6B, and E6B and biotin-labeled G4D. These results indicated that the epitopes for G4D and E6B were not close to each other.

The ELISA absorbance for biotin-labeled B3C was 1.1 when E6B was used as capture antibody. This was between values observed for an epitope distance <3.5 nm (about 0.6) and those for an epitope distance >3.5 nm (about 1.5). These results suggested that steric hindrance occurred and binding of LDH to E6B partially inhibited the subsequent binding of biotin-labeled B3C. Therefore, an intermediate ELISA absorbance was observed. When epitopes are close enough (about 3.5 nm), steric hindrance reportedly would occur, and binding of one antibody partially would prevent binding of a second antibody (Pfeiffer et al., 1987; Wilson, 1991). Thus, the distance between epitopes for B3C and E6B was probably about 3.5 nm. A similar result was also found using B3C as capture MAb and labeled E6B as detection MAb. From the results, the relationship between epitopes for each LDH MAb could be determined, and a model depicting the relative position of each epitope was postulated (Fig. 4).

Detection of turkey muscle in meat

Since binding of MAb D5E did not influence binding of B3C to LDH, an LDH sandwich ELISA with D5E as capture antibody and biotin-labeled B3C as detector antibody was used to quantify turkey muscle in beef and pork. The amount of turkey muscle LDH in beef increased as the content of turkey breast or turkey thigh was increased (Fig. 5). A linear relationship between the amount of turkey muscle LDH and turkey content in beef was observed with correlation coefficients for turkey breast and thigh of 0.992 and 0.975, respectively. A linear relationship between turkey muscle LDH and turkey breast content ($r = 0.992$) and turkey thigh content ($r = 0.991$) in pork was also observed (Fig. 6). These results implied that the amount of turkey muscle LDH measured by sandwich ELISA might be used to estimate the amount of turkey muscle in pork and beef.

The LDH content of turkey thigh was lower than that of turkey breast due to the differential binding ability of MAb D5E to LDH isozymes (Wang et al., 1994). Since the amounts of LDH in turkey breast and turkey thigh are different, the type of turkey muscle must be known for accurate quantification. Nevertheless, LDH MAbs sandwich ELISA could detect low concentrations of turkey muscle in beef and pork. ELISA absorbance for the extracts of beef and pork containing 1% turkey breast or thigh muscle was about six- and threefold higher, respectively, than those containing no turkey. These results indicated LDH MAbs sandwich ELISA could be used to screen for adulteration of raw beef and pork by turkey. This ELISA could also be used to detect adulteration with chicken, as we found in previous studies (Wang et al., 1992; Abouzied et al., 1993) that the LDH antibodies recognized LDH from both chicken and turkey muscle.

REFERENCES

Abouzied, M.M., Wang, C.H., Pestka, J.J., and Smith, D.M. 1993. Lactate dehydrogenase as safe endpoint cooking indicator in poultry breast rolls: Development of monoclonal antibodies and application to sandwich enzyme-linked immunosorbent assay (ELISA). J. Food Prot. 56: 120–124, 129.

Andrews, C.D., Berger, R.G., Mageau, R.P., Schwab, B., and Johnston, R.W. 1992. Detection of beef, sheep, deer, and horse meat in cooked meat products by enzyme-linked immunosorbent assay. J. Assoc. Off. Anal. Chem. 75: 572–576.

Ayob, M.K., Ragab, A.A., Allen, J.C., Farag, R.S., and Smith, C. 1989. An improved, rapid ELISA technique for detection of pork in meat products. J. Sci. Food Agric. 49: 103–116.

Harlow, E. and Lane, D. (Ed.) 1988. *Antibodies, A Laboratory Manual.* Cold Spring Harbor Laboratory, New York.

Ikura, K., Higashiguchi, F., Kitabatake, N., Doi, E., Narita, H., and Sasaki, R. 1992. Thermally induced epitope regions of ovalbumin identified with monoclonal antibodies. J. Food Sci. 57: 635–639.

Jones, S.J. and Patterson, R.L. 1986. A modified indirect ELISA procedure for raw meat speciation using crude antispecies antisera and stabilized immunoreagents. J. Sci. Food Agric. 37: 767–775.

Kaminogawa, S., Shimizu, M., Ametani, A., Hattori, M., Ando, O., Hachimura, S., Nakamura, Y., Totsuka, M., and Yamauchi, K. 1989. Monoclonal antibodies as probes for monitoring the denaturation process of bovine β-lactoglobulin. Biochim. Biophys. Acta 998: 50–54.

Kordossi, A.A. and Tzartos, S. 1987. Conformation of cytoplasmic segments of acetylcholine receptor α- and β-subunits probed by monoclonal antibodies: sensitivity of the antibody competition approach. Eur. Mol. Bio. Organ. J. 6: 1605–1610.

Martin, R., Azcona, J.I., Tormo, J., Hernandez, P.E., and Sanz, B. 1988a. Detection of chicken meat in raw meat mixtures by a sandwich enzyme immunoassay. Int. J. Food Technol. 23: 303–310.

Martin, R., Azcona, J.I., Casas, C., Hernandez, P.E., and Sanz, B. 1988b. Sandwich ELISA for detection of pig meat in raw beef using antisera to muscle soluble proteins. J. Food Prot. 51: 790–794.

Martin, R., Wardale, R.J., Jones, S.J., Hernandez, P.E., and Patterson, R.L.S. 1989. Production and characterization of monoclonal antibodies specific to chicken muscle soluble proteins. Meat Sci. 25: 199–207.

Martin, R., Wardale, R.J., Jones, S.J., Hernandez, P.E., and Patterson, R.L.S. 1991. Monoclonal antibody sandwich ELISA for the potential detection of chicken meat in mixtures of raw beef and pork. Meat Sci. 30: 23–31.

Morgan, M.R.A., Smith, C.J., and Williams, P.A. (Eds.) 1992. *Food safety and quality assurance: Applications of immunoassay systems.* Elsevier Applied Science, New York.

Pfeiffer, N.E., Mehlhaff, P.M., Wylie, D.E., and Schuster, S.M. 1987. Topographical separation of the catalytic sites of asparagine synthetase explored with monoclonal antibodies. J. Biol. Chem. 262: 11565–11570.

Rittenburg, J.H. (Ed.) 1990. *Development and Application of Immunoassay for Food Analysis.* Elsevier Applied Science, New York.

Samarajeewa, U., Wei, C.I., Huang, T.S., and Marshall, M.R. 1991. Application of immunoassay in the food industry. Crit. Rev. Food Sci. Nutr. 29: 403–434.

Smith, A.D. and Wilson, J.E. 1992. Epitopic regions recognized by monoclonal antibodies against rat brain hexokinase: Association with catalytic and regulatory function. Arch. Biochem. Biophys. 292: 165–178.

Smith-Gill, S.J., Lavoie, T.B., and Mainhart, C.R. 1984. Antigenic regions defined by monoclonal antibodies correspond to structural domains of avian lysozyme. J. Immunol. 133: 384–393.

Tzartos, S.J., Rand, D.E., Einarson, B.L., and Lindstrom, J.M. 1981. Mapping of surface structures of electrophorus acetylcholine receptor using monoclonal antibodies. J. Biol. Chem. 256: 8635–8645.

Varshney, G.C., Mahana, W., Filloux, A.M., Venien, A., and Paraf, A. 1991. Structure of native and heat-induced ovalbumin as revealed by monoclonal antibodies: Epitopic changes during heat treatment. J. Food Sci. 56: 224–227, 233.

Wang, C.H., Abouzied, M.M., Pestka, J.J., and Smith, D.M. 1992. Antibody development and enzyme-linked immunosorbent assay for the protein marker lactate dehydrogenase to determine safe cooking end-point temperatures of turkey rolls. J. Agric. Food Chem. 40: 1671–1676.

Wang, C.H., Pestka, J.J., Booren, A.M., and Smith, D.M. 1994. Lactate dehydrogenase, serum protein and immunoglobulin G content of uncured turkey thigh rolls as influenced by endpoint cooking temperature. J. Agric. Food Chem. 42: 1829–1833.

Wilson, J.E. 1991. The use of monoclonal antibodies and limited proteolysis in elucidation of structure-function relationships in proteins. Meth. Biochem. Anal. 35: 207–250.

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Storage Stability of Vacuum Packaged Frozen Pork Sausage Containing Soy Protein Concentrate, Carrageenan or Antioxidants

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ABSTRACT

Fresh pork sausage patties containing carrageenan, without or with soy protein and an antioxidant were packaged with or without vacuum. They were evaluated for sensory properties, visual color, thiobarbituric acid reactive substances (TBARS), and Hunter color 'L', 'a', 'b' values at 4-wk intervals during 16 wk frozen storage. Rosemary extract was as effective as butylated hydroxytoluene (BHT)/propyl gallate (PG)/citric acid (CA) in antioxidant properties, but patties with BHT/PG/CA showed less surface discoloration ($P < 0.05$). In fat-control (FC) products, antioxidants combined with vacuum packaging provided optimum protection against rancidity. With vacuum packaging (VP), reduced-fat products maintained acceptable quality (TBARS and sensory properties) during 16 wks frozen storage.

Key Words: pork sausage, reduced fat, vacuum packaging, antioxidants

INTRODUCTION

OXIDATIVE RANCIDITY is an important factor affecting shelf life of frozen fresh sausage products (Wanous et al., 1989). Lipid oxidation occurring during storage results in disagreeable odor and flavor which lessens the quality. Myoglobin oxidation causes discoloration which influences consumer acceptance. Sherwin (1990) reported that some processed meats, like fresh sausage, were especially oxidation-prone even in frozen storage, where grinding greatly increases exposure of lipids to air. Heme pigments, in meat brought into contact with lipids, are strong oxidation catalysts.

The inclusion of a combination of phenolic antioxidants butylated hydroxytoluene (BHT) or butylated hydroxyanisole (BHA), propyl gallate (PG) and citric acid (CA), is an effective way to decrease oxidation of sausage products (Douglas and Pharlen, 1959; Paul et al., 1968). Hudson (1990) indicated that phenolic antioxidants BHA and BHT were the most common monophenolic antioxidants. Use of BHA has been limited in food products, but BHT has become the choice of phenolic antioxidants. Such antioxidants are soluble in fats and oils, stable at high temperatures, and have synergistic actions with other antioxidants, such as PG, and CA.

Extracts of rosemary are considered an effective alternative to chemical antioxidants. Barbut et al. (1985) indicated that suppression of lipid autoxidation by rosemary oleoresin was more effective than a commercial BHA/BHT/CA blend. Rosemary extracts contain many compounds with antioxidant properties. Four of these, rosmaridiphenol, rosmariquinone, rosmanol and carnosol, were identified as phenolic type compounds (Houlihan et al., 1985), which probably function as free radical scavengers similar to BHA and BHT (St. Angelo et al., 1990).

Effective packaging should maintain the quality of products (Forrest et al., 1975). Polyethylene (PE) bags are commonly used as packaging material due to their low cost and convenience. The usage of PE bags may result in reduced shelf life of meat products because of increased fat oxidation and moisture absorption rate, in comparison to products in vacuum bags which are more costly (Almeida-Dominguez et al., 1992). Vacuum packaging of meat products can decrease rates of oxidation and spoilage compared with oxygen permeable packaging (Newton and Rigg, 1979; Lynch et al., 1986).

Our objective was to investigate the effects of various antioxidants and packaging systems on storage stability and sensory properties of reduced-fat fresh pork sausage products during frozen storage.

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MATERIALS & METHODS

FRESH BONELESS PORK HAMS, picnics and back fat were obtained from a local packer and stored at 4°C overnight before use. The raw materials were trimmed into lean and fat portions and ground separately through a 1.27 cm plate using a Biro Automatic Food Mixer-Grinder (model no. AFMG-48-II), and analyzed for fat content using the modified Babcock method (Pearson and Tauber, 1984). Treatment formulations were: fat-control (FC; conventional product containing 40% fat and 3% added water), carrageenan-control (CC; reduced-fat product containing 9% fat, 0.4% carrageenan and 20% water), and carrageenan-soy product (CS; reduced-fat product containing 9% fat, 0.4% carrageenan, 1.5% soy protein concentrate, and 20% water). Carrageenan (Viscarin SD 389) was obtained from the Marine Colloids Div. of FMC Corp. (Philadelphia, PA); soy protein concentrate (ARCON™ T, F250), from Archer Daniels Midland Co. (Decatur, IL); preblended seasoning (salt 73.5%, dextrose 11.4%, chopped sage 7.3%, black pepper 1.4%, and ground red pepper 6.4%), from A.C. Legg Packing Co., Inc. (Birmingham, AL). Antioxidants were added with other nonmeat raw materials during mixing (2 min) in a Hobart paddle mixer (Model H-120, Hobart Co., Troy, OH). Antioxidant treatments were: no antioxidant products, BHT/PG/CA [products treated with a combination of 0.01% butylated hydroxytoluene (BHT), 0.01% propyl gallate (PG), and 0.01% citric acid (CA) based on fat content], and rosemary extract [treated with rosemary extract (500 ppm based on fat content)]. BHT/PG/CA and rosemary extract were obtained from A.C. Legg Packing Co., Inc. (Birmingham, AL). The products were finely ground through a 0.5 cm plate, made into 50g patties (10 cm dia and 0.5 cm thick) using a Hollymatic Food Portioning Machine (Super 54, Hollymatic Corp., Park Forest, IL) and interleaved with waxed patty papers. Two packaging systems were employed for sausage patties within the same formulation and antioxidant treatment. The patties were divided into 2 groups after freezing and packaging: PE-bag packaging [PE; patties were packaged with oxygen-permeable PE bags] and vacuum packaging [VP; patties were vacuum-packaged using a TurboVac B.V. vacuum packaging machine (Type SB 600, Netherlands) and Cryovac vacuum bags (type 540, 8 × 14 cm, Cryovac Div., W.R. Grace and Co., Simpsonville, SC)]. All sausage patties were individually frozen, packaged 10/bag, placed in cardboard boxes and stored at −20°C until used.

Thiobarbituric acid reactive substances (TBARS)

TBARS analysis procedure of Ke et al. (1984) was used to determine the oxidative rancidity of sausage patties at 0, 4, 8, 12, and 16 wk frozen storage. A standard curve (0 to 2.0×10^{-6} M) was prepared using 1,1,3,3-tetraethoxypropane (TPE) [malonaldehyde bis(diethyl acetal)]. Absorbance readings were taken at 538 nm (Lambda 4A, Model C099-1232 spectrophotometer, Perkin-Elmer, Norwalk, CT) and compared to a standard curve to determine malondialdehyde concentration in samples. TBARS values were expressed as mg malondialdehyde/kg meat.

Table 1—Composition and pH of uncooked fresh pork sausage patties

Traits	Formulation[a]			SEM[b]
	FC	CC	CS	
Moisture	49.8[c]	72.5[d]	70.8[d]	0.08
Fat	34.8[c]	7.7[d]	7.1[d]	0.06
pH	5.94[c]	5.98[d]	6.00[d]	0.008

[a] Formulation codes: FC = regular pork sausage containing 40% fat, and 3% added water; CC = reduced-fat pork sausage containing 7% fat, 0.4% carrageenan, and 20% added water; CS = reduced-fat pork sausage containing 7% fat, 0.4% carrageenan, 1.5% soy concentrate, and 20% added water.
[b] SEM = Standard error of the mean.
[cd] Means within a row with different superscripts differed (P<0.05).

Table 2—Initial sensory properties of fresh pork sausage formulations

Trait[b]	Formulation[a]			SEM[c]
	FC	CC	CS	
Juiciness	6.9[d]	6.0[e]	5.7[f]	0.08
Tenderness	6.9[d]	6.1[e]	6.0[e]	0.07
Off-flavor	6.7[d]	7.0[e]	6.7[d]	0.09
Cohesiveness	5.7[d]	5.5[d]	6.5[e]	0.07
Mealiness	6.4[d]	6.6[e]	6.4[d]	0.07
Flavor intensity	5.9[d]	6.2[e]	6.0[d]	0.07

[a] For formulation codes: see Table 1.
[b] Juiciness, tenderness, off-flavor, cohesiveness, mealiness and flavor intensity were rated on an 8-point scale where 1 = extremely dry, tough, strong, non-cohesive, abundant, bland, and 8 = extremely juicy, tender, none, cohesive, none, intense.
[c] SEM—Standard error of the mean.
[def] Means within a row with different superscripts differed (P<0.05).

pH determination

The pH value of each fresh pork sausage product was determined initially. Fresh pork sausage (10g) from each treatment was homogenized for 30 sec with 100 mL distilled water using an Osterizer (John Oster Mfg. Co., Milwaukee, WI). The pH values of prepared homogenate were determined with a Fisher Accumet (model 805) digital pH meter equipped with a combination electrode.

Proximate analysis

Raw samples were ground twice through a Kitchen Aid Mixer-Grinder (Kitchenaid, Dayton, OH) equipped with a 0.48 cm plate and held at −20°C for subsequent analysis. Moisture and petroleum ether-extractable lipid contents of products were determined in triplicate (AOAC, 1980).

Sensory evaluation

Frozen patties were griddle-broiled on a Special McDonald's grill (Model TG-72, Wolf Range Corp., Compton, CA) at a surface temperature of 165°C for 4.5 min (1.5 min on each side, and another 1.5 min on the first side) until internal temperature 78°C was achieved. Cooked sausage patties were cut into four wedges and held less than 30 min in a 40°C convection oven in metal pans with lids before evaluation by a trained descriptive sensory panel. Training consisted of 30 min sessions where panelists were served sausage patties that varied in attributes to be evaluated. Training sessions were concluded when individual scores did not vary more than one unit from the mean score and panelists were comfortable with the evaluation system. Sausage patties were evaluated for juiciness, tenderness, off-flavor, cohesiveness, mealiness and flavor intensity on an 8-point scale. Samples were coded with random numbers and one wedge of each treatment was served to panelists in a random manner. Panelists were seated in individual booths under red light to mask visual differences between samples (AMSA, 1978). Each treatment replication was evaluated initially and at 4, 8, 12, and 16 wks.

Color evaluation

Frozen raw sausage patties were allowed to equilibrate at room temperature (≈23°C) for 30 min, overwrapped with an oxygen permeable plastic film (Handi-Wrap II, Dow Consumer Products Inc., Indianapolis, IN) covering the exposed surface and then held an additional 30 min at 6–8°C prior to evaluation. A trained, 14-member color panel was used for visual color determination. Patties were evaluated for overall color (8 = extremely red and 1 = extremely brown) and for percent discoloration (0 to 100%) under warm white fluorescent lighting positioned to provide 70 lumens at the counter surface. Training consisted of 30 min sessions where panelists visually examined sausage patties that varied in attributes to be evaluated. Training sessions were concluded when individual scores did not vary more than one unit from the mean score and panelists were comfortable with the evaluation system. Two patties of each treatment were evaluated for Hunter color 'L', 'a', and 'b' values using the Hunter Lab Digital Color Difference Meter (Model D25D2A, Reston, VA) following visual color determination. The Hunter Color meter was standardized with a pink standard plate (L = 68.7, a = 23.0, b = 9.4). Each treatment replication was evaluated initially and at 4, 8, 12, and 16 weeks in duplicate.

Statistical analysis

Statistical model used in the study was a split-plot design with three replications (Steel and Torrie, 1980). The main plot was represented by 18 treatment combinations, arranged as a 3 × 3 × 2 factorial consisting of three formulations (FC, CC, and CS), three types of antioxidants (no antioxidant, BHT/PG/CA, and rosemary extract), and two packaging systems (PE-bag and vacuum packaging). The subplots were represented by 5 storage periods (0, 4, 8, 12, and 16 wks). Data were analyzed using analysis of variance by the general linear models procedure (GLM) of Statistical Analysis System (SAS Institute, Inc., 1988). Least Square Means was employed to separate the means (Steel and Torrie, 1980) when analysis of variance indicated a significant ($P < 0.05$) effect.

RESULTS & DISCUSSION

Composition and pH

Compositions and pH analyses of raw fresh pork patties (Table 1) showed antioxidants and packaging systems did not affect initial compositions and pH ($P > 0.05$). FC contained the highest amount of fat and the lowest moisture ($P < 0.05$). No differences ($P > 0.05$) occurred in the composition of reduced-fat products. The pH values were higher for reduced-fat sausage patties than for FC ($P < 0.05$) which may have resulted from the basic properties of carrageenan (FMC, 1988). No differences occurred in pH values between CC and CS ($P > 0.05$).

Sensory properties

Antioxidant and packaging system did not affect ($P > 0.05$) initial sensory properties of fresh pork sausage products. FC had a higher ($P < 0.05$) juiciness score than CC and CS (Table 2). Significant interaction means for juiciness between packaging system and storage time (Table 3) indicated that juiciness scores for patties packaged in PE declined faster than those packaged in VP ($P < 0.05$). This may be the result of higher moisture losses in PE than in VP during storage. Reagan et al. (1983) reported that juiciness scores for fresh pork sausage patties packaged in PVC film declined after 21 days frozen storage (−18°C). Matlock et al. (1984) also reported that VP improved juiciness scores over sausage patties wrapped in PVC film. Tenderness values (Table 2 and 4) were related to both formulations and storage time ($P < 0.05$). FC patties were more tender than CC and CS (Table 2). Tenderness scores declined ($P < 0.05$) during extended frozen storage (Table 4). Matlock et al. (1984) also reported this trend and explained that it may be due to the concomitant decline in juiciness. CC had less detectable off-flavor than FC and CS (Table 2), indicating panelists detected more off-flavor in the higher fat and soy protein concentrate treatments. Significant interaction means ($P < 0.05$) for off-flavor scores, stratified by formulation and storage time (Table 5) showed FC had lower off-flavor scores (more off-flavor) after 12 wks ($P < 0.05$), while CS reached the same level off-flavor as FC at 16 wks. However, reduced-fat patties made with carrageenan had less perceptible off-flavor through the end of the study (16 wks). The soy protein concentrate may result in off-flavor in reduced-fat sausage products after long term storage. Significant interaction means of storage time and packaging system (Table 3) indicated that the initial off-flavor score did not differ ($P > 0.05$) between various packaging systems. After 4-wk storage, VP significantly decreased incidence of off-flavor ($P < 0.05$). At 12-wk storage, PE packaged patties had lower

Table 3—Packaging effects on sensory and color traits during frozen storage

	Storage time (wk)										
	0		4		8		12		16		
Traits	PE[h]	VP	PE	VP	PE	VP	PE	VP	PE	VP	SEM[i]
Juiciness	6.2[b]	6.2[b]	5.9[c]	6.2[b]	5.9[e]	6.2[b]	5.8[c]	6.0[f]	5.7[d]	6.0[f]	0.07
Off-flavor	6.8[c]	6.7[c]	6.7[c]	6.9[b]	6.7[c]	7.0[b]	6.4[d]	6.7[c]	6.1[e]	6.5[d]	0.07
Cohesiveness	6.2[b]	6.1[c]	6.0[d]	6.1[c]	6.1[c]	6.2[b]	6.1[c]	6.2[b]	5.8[e]	6.2[b]	0.05
Discoloration	7.5[b]	8.5[bc]	10.0[c]	12.5[d]	17.0[c]	18.0[ef]	19.5[f]	17.5[e]	26.0[g]	25.0[g]	0.58

[a] Traits: Juiciness, off-flavor, and cohesiveness; see Table 2, Discoloration; (0=no discoloration, 100=full discoloration).
[bcdefg] Means within a row with different superscripts differed (P<0.05).
[h] Package codes: PE = oxygen-permeable polyethylene bags; VP = vacuum packaging.
[i] SEM = standard error of the mean.

Table 4—Effects of storage time on tenderness, flavor intensity and Hunter color 'L' values

	Storage time (wk)					
Traits[a]	0	4	8	12	16	SEM[e]
Tenderness	6.3[b]	6.2[bc]	6.2[bc]	6.1[cd]	6.0[d]	0.05
Flavor intensity	6.1[b]	6.1[b]	6.1[b]	5.8[c]	5.9[c]	0.04
Hunter 'L'	47.9[b]	47.0[b]	45.8[c]	45.6[c]	44.6[c]	0.32

[a] Traits: Tenderness and flavor intensity; see Table 2, Hunter Color value; 'L' = lightness/darkness (0 = black, 100 = white).
[bcd] Means within a row with different superscripts differed (P<0.05).
[e] SEM = standard error of the mean.

Table 5—Effects of formulation and antioxidants on off-flavor, cohesiveness, and color during frozen storage

	Storage time (wk)					
Traits[a]	0	4	8	12	16	SEM[i]
Off-flavor						
FC[b]	6.7[e]	6.7[e]	6.8[e]	6.2[f]	6.2[f]	0.09
CC	7.1[d]	6.8[e]	6.8[e]	6.7[e]	6.5[e]	
CS	6.7[e]	7.0[de]	6.8[e]	6.5[e]	6.1[f]	
Cohesiveness						
FC	5.7[d]	5.5[e]	5.8[d]	5.8[d]	5.9[f]	0.07
CC	6.5[g]	6.3[h]	6.3[h]	6.3[h]	6.1[i]	
CS	6.5[g]	6.3[h]	6.3[h]	6.3[h]	6.1[i]	
Hunter "b"						
FC	14.5[d]	14.2[de]	13.6[e]	14.2[de]	14.2[de]	0.16
CC	12.0[f]	11.7[f]	9.0[g]	12.0[f]	11.8[f]	
CS	12.0[f]	11.7[f]	8.6[g]	11.8[f]	11.8[f]	
Overall color						
N[c]	6.5[e]	6.0[f]	5.0[g]	5.0[g]	5.0[g]	0.11
B	7.0[d]	6.0[f]	5.0[g]	5.0[g]	5.0[g]	
R	6.1[f]	6.0[f]	5.0[g]	5.0[g]	5.0[g]	
Hunter "a"						
N	8.5[d]	6.8[f]	6.8[f]	6.6[f]	6.0[g]	0.16
B	8.9[d]	7.4[e]	7.2[ef]	6.8[f]	6.4[f]	
R	9.0[d]	6.8[f]	7.4[e]	6.7[g]	6.2[g]	

[a] Traits: Off-flavor, and cohesiveness; see Table 2, Hunter color values; 'a' = redness/greeness (+ = red, − = green), 'b' = Yellowness/blueness (+ = yellow, − = blue), overall color; (8 = extremely red, and 1 = extremely brown).
[b] For formulation codes: see Table 1.
[c] Antioxidant codes: N = no antioxidants, B = combination of 0.01% butylated hydroxytoluene (BHT), 0.1% propyl gallate (PG), and 0.01% citric acid based on fat content, R = rosemary extractive (500 ppm based on fat content).
[defghi] Means within an evaluation trait with different superscripts differed (P < 0.05).
[i] SEM = standard error of the mean.

off-flavor scores (P < 0.05) than at 0, 4, and 8 wk. From 4–16 wk storage, VP decreased development of off-flavor (P < 0.05). Starting at 4 wk frozen storage, PE sausage patties had less off-flavor than those in VP (P < 0.05). Thus, VP was less effective than PE in preventing development of off-flavor. This disagreed with results of Lynch et al. (1986) working with storage stability of ground beef. CS were more (P < 0.05) cohesive than FC or CC (Table 2). Cohesiveness scores decreased and then increased (P < 0.05) in FC as storage time increased (Table 5). In contrast, reduced-fat products (CC and CS) decreased in cohesiveness over storage (P < 0.05). Patties packaged in VP were more (P < 0.05) cohesive than those packaged in PE beyond 8 wks (Table 3). CC were less (P < 0.05) mealy than FC or CS (Table 2). Packaging systems and storage time did not affect mealiness (P > 0.05). CC had higher (P < 0.05) flavor intensity than FC and CS (Table 2). The flavor intensity scores of fresh pork sausage patties were affected (P < 0.05) after 8 wks frozen storage (Table 4).

TBARS

TBARS values are an indicator for development of oxidative rancidity in meat products. Initial TBARS values indicated no differences (P > 0.05) among formulations (Fig. 1). A significant interaction of TBARS values occurred among formulations, antioxidants, packaging systems and storage times (P < 0.05) in fresh pork sausage patties (Fig. 1). In general, TBARS values for all treatments increased over storage (P < 0.05). TBARS were different (P < 0.05) between products without or with antioxidant. In patties without antioxidant, TBARS in reduced-fat products were lower (P < 0.05) than FC within a given packaging system. Patties in VP had lower (P < 0.05) TBARS than those packaged in PE with the same formulation. In products containing BHT/PG/CA, TBARS were <1 mg malondialdehyde/kg meat after 16 wks frozen storage regardless of formulation or packaging system. Products containing rosemary extract had TBARS <1 mg malondialdehyde/kg meat throughout storage, with exception of FC in PE packaging at 16 wks. BHT/PG/CA and rosemary extract were effective in retarding oxidation of fresh pork sausages. TBARS values for the regular (FC) fresh pork sausages increased rapidly (P < 0.05) without added antioxidant. However, in FC without antioxidants, VP (P < 0.05) retarded oxidative rancidity, but TBARS values were still higher than those of products containing antioxidants (P < 0.05). Thus, addition of antioxidants was effective in preventing lipid oxidation in high-fat sausage products. In CC products, the rate of oxidation was slower than FC. For VP patties, TBARS of CC were lower than those of FC throughout frozen storage. CS had higher (P < 0.05) TBARS than CC at 8-wks storage (P < 0.05). Methods of packaging can affect TBARS values in sausage products over storage. However, if products contain a high amount of fat and have no antioxidant added, VP alone is not a suitable replacement for antioxidants in high fat products. With reduced-fat patties, VP alone aided in retarding oxidative rancidity during frozen storage.

Visual evaluation

A significant interaction was found for overall color scores among formulations, packaging systems and storage times (Fig. 2). Overall color scores decreased as storage time increased with a change from reddish-pink to grayish-brown. FC patties had less desirable color over the storage period than either CC or CS (Fig. 2). VP resulted in lower color scores at week 4 for CC and CS, but higher color scores at 8–16 wk storage compared to PE. CC and CS visual color scores within packaging treatment were similar from 8 through 16 wk storage. Overall color scores for FC were not different (P > 0.05) between packaging systems nor did color scores differ for reduced-fat treatments between formulations (data not shown). BHT/PG/CA added products had higher (P < 0.05) initial overall color scores than other products (Table 5). However, after 4 wks no differences occurred in overall color scores among patties with or without antioxidants (P > 0.05). Lipid oxidation in muscle tissue has

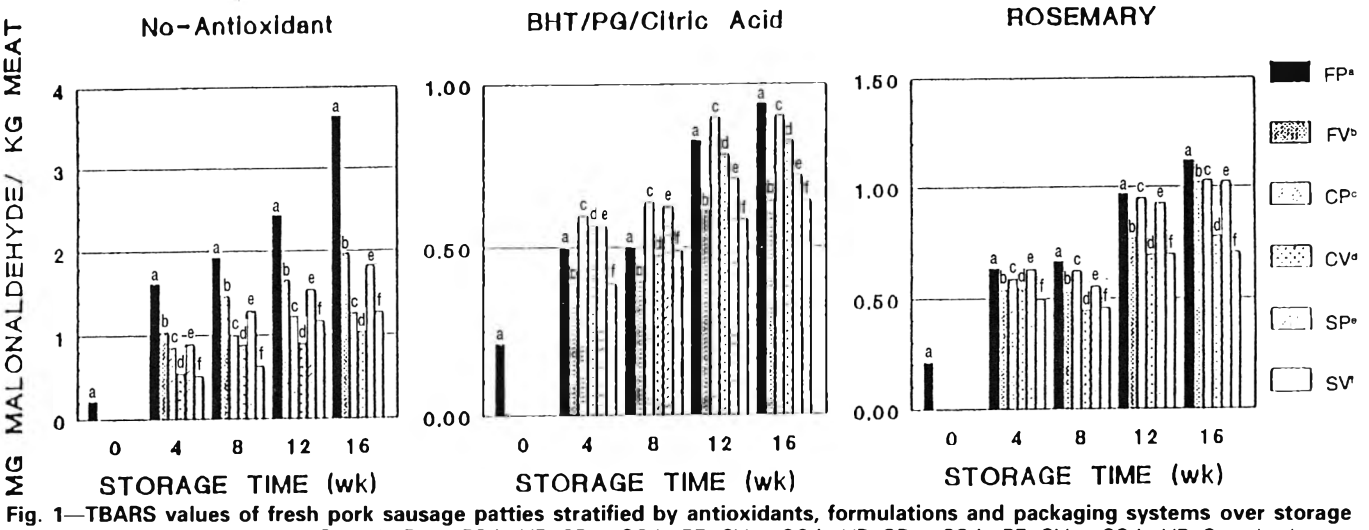

Fig. 1—TBARS values of fresh pork sausage patties stratified by antioxidants, formulations and packaging systems over storage time. Treatment codes: FP = FC in PE; FV = FC in VP; CP = CC in PE; CV = CC in VP; SP = CS in PE; SV = CS in VP. Standard error of the mean = 0.119.

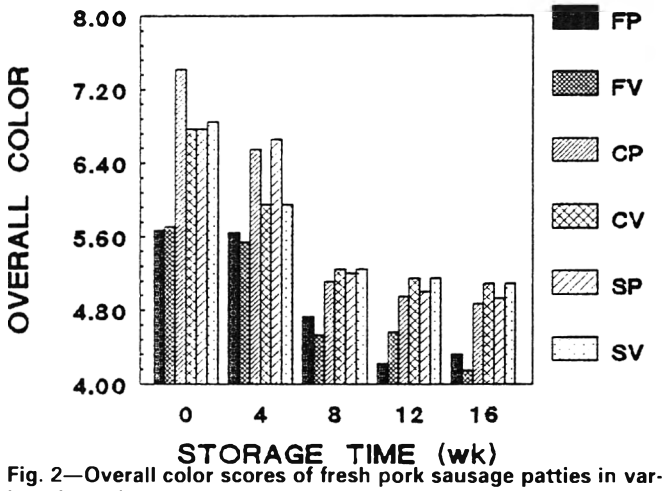

Fig. 2—Overall color scores of fresh pork sausage patties in various formulations and packaging systems over storage time. For treatment codes: see Fig. 1. Standard error of the mean = 0.16.

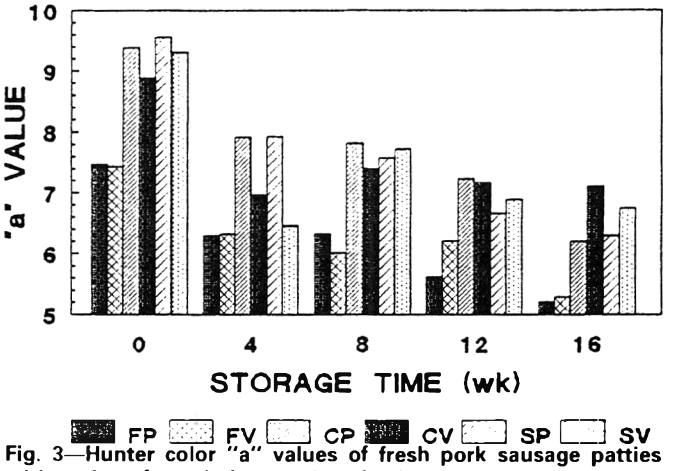

Fig. 3—Hunter color "a" values of fresh pork sausage patties with various formulations and packaging systems over storage time. For treatment codes: see Fig. 1. Standard error of the mean = 0.22.

been shown to enhance meat discoloration (Watts, 1962; Greene, 1969). The products with BHT/PG/CA were lower ($P < 0.05$) in discoloration than with no-antioxidant added and rosemary extract added products (15.5 vs 16.8 and 16.3) regardless of formulations and packaging systems. The discoloration of products with rosemary extract were not different from those products without antioxidant ($P > 0.05$). The discoloration percentages of VP products were higher than those of products packaged in PE at 4 wk storage period (Table 3). After 4 wk, discoloration increased ($P < 0.05$) regardless of packaging system.

Hunter color evaluation

Regular pork sausage patties (FC) had higher ($P < 0.05$) Hunter color 'L' (lightness) values (54.26) than CC (42.07) and CS (42.48) probably because of greater fat content in the FC product (data not shown). There was a decline ($P < 0.05$) in Hunter color 'L' values over time (Table 4). No significant differences in Hunter color 'L' values were observed up to 4 wk frozen storage. Surface color of sausage patties became darker as storage increased. Generally, all treatments decreased ($P < 0.05$) in 'a' values over storage times (Fig. 3). Reduced-fat products were redder than FC patties due to greater lean content. Hunter 'a' values decreased after 4 wk and then declined gradually over 16 wk storage in most reduced-fat products (except CC with vacuum packaging). Initial Hunter color 'a' values were not different ($P > 0.05$) among treatments with or without antioxidants (data not shown). However, at 4 wk storage, treatments with antioxidant BHT/PG/CA were higher ($P < 0.05$) in Hunter color 'a' values than the others (Table 5). Hunter color 'a' values decreased as storage time progressed. The differences among antioxidant groups were not significant at 0, 8, 12 and 16 wk storage ($P > 0.05$). FC had higher ($P < 0.05$) Hunter color 'b' (yellowness) than reduced-fat patties (Table 5). Hunter color 'b' values in all formulations declined ($P < 0.05$) at 8 wks and then increased to original levels. There was no difference between CC and CS in Hunter color 'b' values. A significant interaction for Hunter color 'b' values in antioxidants along with packaging systems occurred (Fig. 4).

CONCLUSIONS

IN CONVENTIONAL sausage products which contained a higher level of fat, antioxidants were needed to prevent development of oxidative rancidity, while, in reduced-fat products, vacuum packaging (VP) maintained quality of fresh pork sausage during 16 wk frozen storage. Reduced fat sausage patties containing

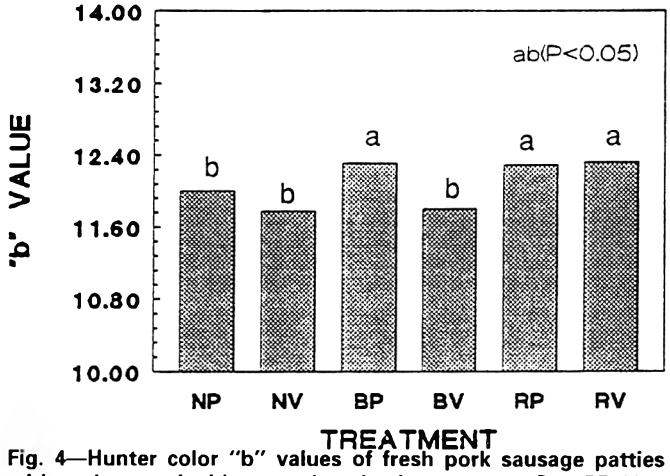

Fig. 4—Hunter color "b" values of fresh pork sausage patties with various antioxidants and packaging systems. P = PE; V = VP; N = no antioxidants; B = BHT/PG/CA; R = rosemary extract. Bars with different letters differed ($P < 0.05$). Standard error of the mean = 0.20.

carrageenan and water (CC) had higher flavor intensity scores and less detectable off-flavor than regular pork sausage (FC) or reduced fat sausage containing soy concentrate and water (CS).

REFERENCES

Almeida-Dominguez, N.G., Higuera-Ciapara, I., Goycoolea, F.M., and Valencia, M.E. 1992. Pack, temperature and TBHQ effects on oxidative deterioration of corn-based snacks. J. Food Sci. 57: 112–117.

AMSA. 1978. *Guidelines for Cookery and Sensory Evaluation of Meat.* Am. Meat Sci. Assoc. in cooperation with Nat. Live Stock and Meat Board, Chicago, IL.

AOAC. 1980. *Official Methods of Analysis*, 13th ed. Association of Official Analytical Chemists, Washington, DC.

Barbut, S., Josephson, D.B., and Maurer, A.J. 1985. Antioxidant properties of rosemary oleoresin in turkey sausage. J. Food. Sci. 50: 1356–1359, 1363.

Douglas, L.R. and Pharlen, W.E. 1959. Method of protecting dry sausage against discoloration and rancidity. U.S. Patent 2,901,354, August 25, 1959.

FMC. 1988. Marine colloids application bulletin—The carrageenan people. Number b-69. FMC Corp., Marine Colloids Div., Philadelphia, PA.

Forrest, J.C., Aberle, E.D., Hedrick, H.B., Judge, M.D., and Merkel, R.A. 1975. Methods of storing and preserving meat. Ch. 11. In *Principles of Meat Science*, p. 285. W.H. Freeman and Company, New York.

Greene, B.E. 1969. Lipid oxidation and pigment changes in raw beef. J. Food Sci. 34: 110–113.

Houlihan, C.M., Ho, C.T., and Chang, S.S. 1985. The structure of rosmariquinone—a new antioxidant isolated from rosmarinus officinalis L. J. Am. Oil Chem. Soc. 62: 96–98.

Hudson, B.J.F. 1990. *Food Antioxidants.* Elsevier Appl. Sci. Pub. Co., Inc., New York.

Ke, P.J., Cervantes, E., and Robles-Martinez, C. 1984. Determination of thiobarbituric acid reactive substances (TBARS) in fish tissue by an improved distillation-spectrophotometric method. J. Sci. Food Agric. 35: 1248–1254.

Lynch, N.M., Kastner, C.L., Caul, J.F., and Kropf, D.H. 1986. Flavor profiles of vacuum packaged and polyvinyl chloride packaged ground beef: A comparison of cooked flavor changes occurring during product display. J. Food Sci. 51: 258–262, 267.

Matlock, R.G., Terrell, R.N., Savell, J.W., Rhee, K.S., and Dutson, T.R. 1984. Factors affecting properties of raw-frozen pork sausage patties made with various NaCl/phosphate combinations. J. Food Sci. 49: 1363–1366, 1371.

Newton, K.G. and Rigg, W.J. 1979. The effect of film permeability on the storage life and microbiology of vacuum-packaged meat. J. Appl. Bacteriol. 47: 433–441.

Paul, D.L., Griesbach, R.H., and Jaeger, J.F., 1968. Method of preserving fresh frozen pork sausage. U.S. Patent 3,366,495, Jan. 30.

Pearson, A.M. and Tauber, F.W. 1984. Analytical methods, Ch. 16, In *Processed Meats*, 2nd ed., p. 351. Van Nostrand Reinhold Co., Reinhold, New York.

Reagan, J.O., Liou, F.H., Reynolds, A.E., and Carpenter, J.A. 1983. Effect of processing variables on the microbial, physical and sensory characteristics of pork sausage. J. Food Sci. 48: 146–149, 162.

SAS Institute, Inc. 1988. *SAS USER'S Guide., Statistics.* Statistical Analysis System Institute, Inc., Cary, NC.

Sherwin, E.K. 1990. Antioxidant. Ch. 5. In *Food Additives*, A.L. Branen, P.M. Davidson, and S. Salminen (Ed.). Marcel Dekker, Inc. New York.

St. Angelo, A.J., Crippen, K.L., Dupuy, H.P., and James, C., Jr. 1990. Chemical and sensory studies of antioxidant-treated beef. J. Food Sci. 55: 1501–1505, 1539.

Steel, R.G.D. and Torrie, J.H. 1980. *Principles and Procedures of Statistics*, 2nd ed. McGraw-Hill Book Co., New York.

Wanous, M.P., Olson, D.G., and Kraft, A.A. 1989. Oxidative effects of meat grinder wear on lipids and myoglobin in commercial fresh pork sausage. J. Food Sci. 54: 545–548, 552.

Watts, B.M. 1962. Meat products. In *Lipids and Their Oxidation in Foods*, H.W. Schultz, E.A. Day, and R.O. Sinnhuber (Ed.), p. 202. AVI Pub. Co., Westport, CT.

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Pressure-Induced Dimerization of Metmyoglobin

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ABSTRACT

Pressure treatment at 7.5 kbar of solutions of metmyoglobin led, as shown by electrophoresis and chromatographic analysis, to the formation of spectrally similar monomeric and dimeric forms. The dimer was formed in the pH range 6–10, though maximum formation was around the isoelectric point (pH 6.9). At acid pH no dimer or higher oligomers formed. In sodium chloride (0.5 to 2M), at neutral pH lower concentrations of dimer formed than in its absence. The dimer was stabilized by SDS-labile linkages and had a slightly lower thermal denaturation temperature than the monomer. If similar changes occur with other proteins, then food quality may be modified by pressure treatments.

Key Words: meat, metmyoglobin, high pressure, dimerization

INTRODUCTION

THE EFFECT OF HIGH PRESSURE on biological systems has been studied for almost 100 years (Hite, 1899). High pressure can bring about microbial destruction and markedly affect the rate of enzymic reactions (Morild, 1981; Gould, 1994) as well as modifying the rate of chemical reactions. In addition, it may cause conformational changes in proteins if these are associated with a decrease in volume (Morild, 1981).

Interest has been further stimulated by the commercial possibility of applying high pressure to preserve foods without undesirable sensory changes often associated with thermal processing. Thus research is undertaken on kinetics and mechanisms of pressure-induced unfolding of proteins, and also on subsequent aggregation reactions. These lead to formation of gels and precipitates and may greatly affect acceptability, especially textural quality of the food. Post-pressure effects are also important, since foods would be stored prior to consumption. When a protein is subjected to high pressure the total charge would probably increase since ionization usually involves decrease in volume (Isaacs, 1981). Such charged groups would be hydrated but still may affect both unfolding of the protein and its subsequent aggregation. Hydrophobic interactions would rupture under pressure (Morild, 1981), while covalent linkages should resist rupture. The fate of hydrogen bonds is less predictable in biopolymer systems (Morild, 1981). If changes in conformation occur following pressure treatment, then eating quality and shelf life of a food containing such proteins would be affected since their enzymic, chemical and or physical properties would change. Metmyoglobin, as a well characterized protein, was chosen for further study.

In agreement with earlier studies by Zipp and Kauzmann (1973), we showed (Defaye et al., 1995) that, when metmyoglobin was denatured by pressures of 7–8 kbars, it rapidly 'renatured' at all pHs except near the isoelectric point (pH 6.9). At those pH values a precipitate formed. However, the precipitate behaved very differently from the precipitate induced by thermal treatment. On standing it redissolved to produce a solution with spectral properties similar to native metmyoglobin. However, the nature of the resolubilized heme pigment was not determined. Our objective was to determine the nature of the renatured soluble metmyoglobin formed following pressure-denaturation.

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MATERIALS & METHODS

HORSE HEART METMYOGLOBIN (LD+61H7106, Sigma Chemical Company, St. Louis, MO) was used without further purification. All solutions were prepared with deionized, distilled water and pH adjustment was by appropriate concentrations of HCl or NaOH. All other reagents used were analytical grade.

Pressure application was at 7.5 kbar and ambient temperature (≈23°C) for 20 min. Such conditions cause metmyoglobin to denature even at neutral pH (Defaye et al., 1994). The pressurized samples were left to renature for 3 days at 5°C before analysis. At all pH values >97% solubilization occurred under these conditions.

Electrophoretic analysis

Native and sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed in gels of 12.5% concentration (Andrews, 1988; Laemmli, 1970). Solutions were diluted with 0.125 M Tris buffer (pH 6.8) to give a concentration of 1 mg/mL and 10 µg of protein was applied to each well. Fixation of the protein was by 12% trichloroacetic acid for 1 hr and staining using Coomassie brilliant blue G-250 (Neuhoff et al., 1988). Gels were scanned at 580 nm using a Shimadzu cs-9000 dual-wavelength flying spot scanner.

Sephadex chromatography

Samples (200 µL of 0.2% native or pressure-treated metmyoglobin) were fractionated on Sephadex G-100 (bed volume 120 cm^3, height 60 cm and flow rate 25 $ml \cdot h^{-1}$) in 0.05 M phosphate buffer, pH 6.8. Absorbance of the eluant was monitored at 220 nm. The column was calibrated with lysozyme (14,300), ovalbumin (45,000), trypsinogen (24,000) and metmyoglobin (17,000). In some experiments, where greater volumes for subsequent analysis were required, the sample volume was increased to 400 µL.

UV/visible spectrophotometry and differential scanning calorimetry

Spectra were recorded against distilled water in 1-cm cells at a scan rate of 20 nm. min^{-1} (Perkin-Elmer Lambda 5). Thermograms were obtained using a Perkin Elmer DSC-7 calibrated with indium and zinc. Samples of 5% pressurized or native metmyoglobin (30 ± 2 mg of the solution) were weighed (to 0.01 mg) into 50-µL standard aluminum pans and sealed. Samples were heated from 40–90°C at 5°C/min with an empty, sealed pan as reference. Onset temperature, first deflection from the baseline (To) and peak temperature (Tp) were measured.

Protein-to-heme ratio

After concentrating the fractions from Sephadex chromatography by freeze-drying and redissolving in 3 mL deionized water, protein was determined by the Sigma Diagnostics (1991) modified biuret method and heme according to a modification of the method of Labbe et al., (1979).

RESULTS

Spectral analysis

The visible spectra of pressure-treated metmyoglobin at three pH values (4.0, 6.8, and 9.0) were identical to those of native proteins indicating the heme environments had not been modified to any notable extent following pressure treatment.

Table 1—Proportion of slower moving component following pressure treatment of 0.2% MetMb at different pH's (means ± SD of 4 runs)

Initial pH[a]	Concentration (%)
4.0	0
5.0	0
6.0	9.6 ± 2.0
6.8	16.8 ± 1.5
7.0	18.3 ± 2.0
7.5	18.1 ± 3.0
8.0	15.7 ± 5.0
9.0	10.2 ± 4.0
10.0	7.5 ± 4.0

[a] Final pH values are not quoted since they were variable. At around pH 4–5 they increased by 0.2 to 0.7 units, at pH 7 there was little change (± 0.3 pH unit) and at pH 9–10 they decreased by 0.2 to 0.9 units.

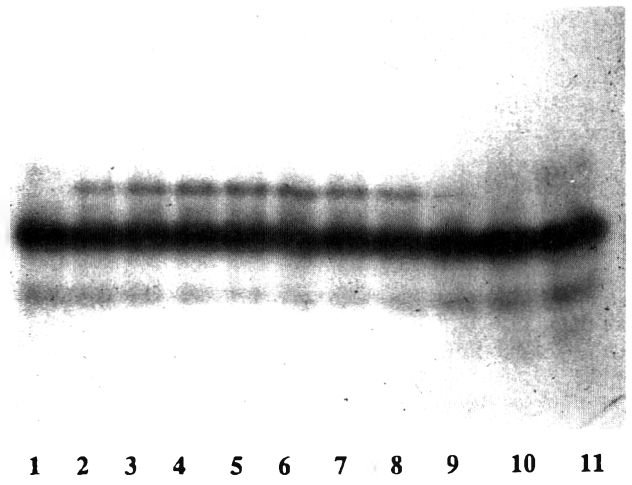

Fig. 1—Typical native PAGE analysis (cathode top) of 1.2×10^{-4} M MetMb pressurized at different pH values and allowed to renature for 3 days at 5°C. Lane 1, native; Lane 2, pH 9; Lane 3, pH 8; Lane 4, pH 7.5; Lane 5, pH 6.8; Lane 6, pH 7; Lane 7, pH 6.5; Lane 8, pH 6; Lane 9, pH 5; Lane 10, pH 4; and Lane 11, native.

Electrophoretic behavior

Effect of pH and salt. Since no buffers were used, changes in pH occurred in some samples (Table 1) and thus all pHs indicated are initial pre-pressure-treated values. PAGE electrophoretograms at pH 6.8 of native and renatured metmyoglobin following pressure treatment at pH 4–10 were compared (Fig. 1). The electrophoretograms indicated pH affected the nature of the renatured products. At pH 5 and below, one band occurred which migrated at the same rate as the native protein. At pH 6 and above, two bands occurred, one migrating with the same mobility as native metmyoglobin and one more slowly. The proportions of the two components were estimated by gel scanning (Table 1). The maximum concentration of the slower moving component appeared to be formed around the isoelectric point of metmyoglobin, i.e., pH 6.9.

On one occasion, samples at pH 7–8 had further slower moving bands of low concentration indicating possible formation of higher oligomeric products. No explanation could be found to explain this one anomalous result.

Pressure treatment in sodium chloride solutions (0.5 to 2M) at pH 6.8 yielded mixtures of the same two components found in the absence of salt. The relative concentrations of the slower moving component were 4.5, 10.0 and 9.9% of the total concentration value at 0.5, 1.0 and 2.0M salt (single run). When electrophoresis was carried out in 1% SDS or a mixture of 1% SDS plus 1% β-mercaptoethanol no difference occurred between native protein and any pressure-treated samples.

Effect of concentration on ratio of the two components. Different concentrations (0.2–20%) of metmyoglobin were pressurized at pH 6.8 and PAGE electrophoresis of the samples was performed at that pH. The proportion of each component was estimated (Table 2). An increase in concentration of the slower moving component appeared with increasing total concentration. No additional bands were observed at 0.2% in any samples.

Table 2—Proportion of slower moving component following pressure treatment of metMb at pH 6.8 (means ± SD of 3 runs)

MetMb concentration (%)	Concentration (mean ± SD of 3 runs)
0.2	16.8 ± 1.5[a]
0.6	23.3 ± 4.0
1.2	18.7 ± 2.4
2.5	23.8 ± 0.4
5.0	27.2 ± 2.0
10.0	33.1 ± 6.0
20.0	30.5 ± 3.0

[a] Means of four runs.

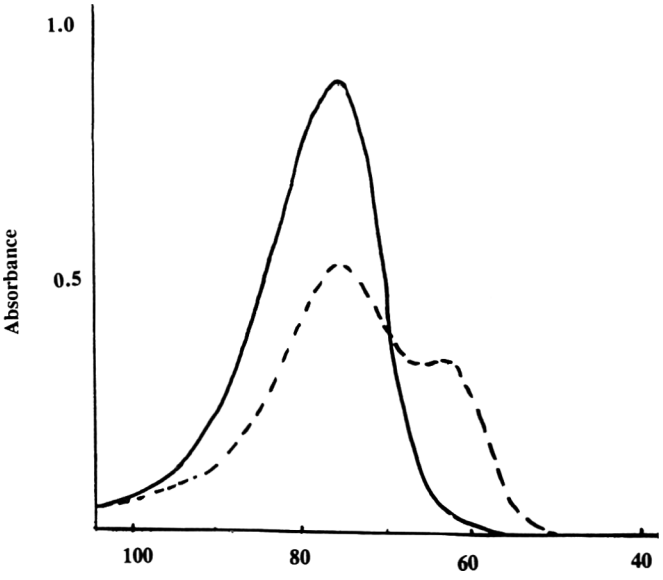

Fig. 2—Elution pattern of 1.2×10^{-4} M native (—) and pressure-treated (---) MetMb on a Sephadex G-100 column with pH 6.8 phosphate buffer. 200 µL of the native or renatured MetMb at pH 6.8 was applied to the column. Calibration proteins eluted at 67.5 mL (ovalbumin, 45,000), 82.5 mL (trypsinogen, 24,300) and 104 mL (lysozyme, 14,300). Native metmyoglobin (17,000) eluted at 87.5 mL and the 'dimer' at 72.5 mL (estimated M W = 34,000).

Sephadex chromatography

Sephadex chromatography of native and renatured material demonstrated that renatured material had two distinct components as compared to the one from native protein (Fig. 2). They had molecular weights of about 17000, i.e., equivalent to native metmyoglobin, and about 34000. This indicated that the slower moving component was a dimer of metmyoglobin, stabilized by SDS-labile bonds.

Differential scanning calorimetry

DSC analysis indicated little difference between unpressurized and pressurized, resolubilized samples at pH 6.8 (Fig. 3). The peak temperatures for renatured and native materials were the same (75.5 ± 0.2°C and 75.7 ± 0.6°C), but onset temperatures were lower for pressure-treated samples (65.5 ± 0.8°C) than for native material (68.7 ± 0.8°C). This suggested that the dimer (about 27% of the mixture, Table 2) was of slightly lower thermal stability than the monomer. Values are means (± standard deviation) of 7 determinations.

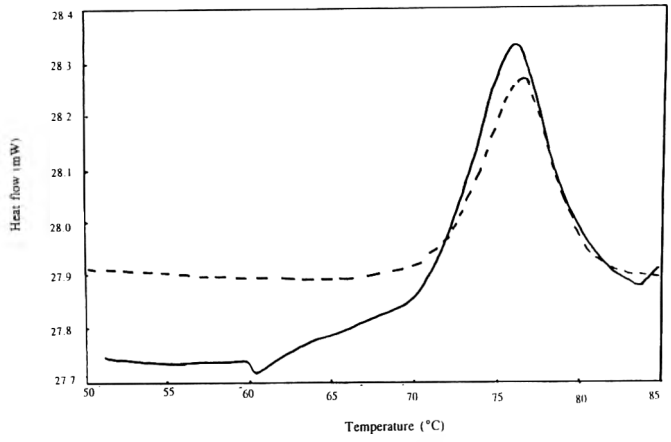

Fig. 3—Thermograms obtained at 5°C min^{-1} of 30.29 mg of 5% native MetMb (dashed line) and 31.92 mg of 5% pressure-treated MetMb (solid line). Initial pH = 6.8 in both cases.

Analysis of individual components

The two components from the pressure-treated system were separated on Sephadex G 100 and subjected to both spectrophotometric and chemical analysis. The UV/VIS spectra were identical and typical of metmyoglobin. Analysis of heme and protein contents of the fractions and native metmyoglobin indicated relative ratios were the same, i.e., each apomyoglobin chain had one heme group.

DISCUSSION

SEVERAL REPORTS have indicated commercially prepared samples of metmyoglobin had a small amount of dimer. It has been suggested that this dimer was formed during freeze-drying (van den Oord et al., 1969). Gamma-irradiation of metmyoglobin can also induce dimerization (Lycometros and Brown, 1973). However, in both cases the dimer appeared to be stabilized by non-disulfide covalent bond(s) since it resisted dissociation by SDS, urea and β-mercaptoethanol. The dimer produced by high-pressure treatment of metmyoglobin leads to formation of a dimer on return to normal pressure. This was presumably stabilized by hydrophobic and/or hydrogen bonds since it was dissociated in SDS. The observation that salt had little effect suggested electrostatic forces were not important in stabilizing the dimer, though the slightly lower concentration of dimer found in the salt solution suggests charge effects may have an indirect effect. Thus, the dimer produced on pressure treatment differed from those produced by drying or irradiation. Interestingly, in all commercial samples of metmyoglobin analyzed, no evidence for any component other than native monomer was found.

We also studied the renatured forms of metmyoglobin subjected to both acid and urea denaturation at atmospheric pressure. On reducing pH of a metmyoglobin solution to 2.5 at room temperature and subsequent renaturation at pH 7, a single component of identical electrophoretic mobility to native material formed. In addition, adjustment to pH 12 and subsequent reduction to pH 7 at normal pressure and room temperature yielded a single component with identical mobility to native protein. Following urea denaturation at neutral pH (8M) and renaturation by dialysis, only the monomeric, native protein was formed. The formed dimers appeared to be resistant to small changes in pH since reducing pH in solutions where both monomers and dimers were present to pH 5 still yielded both forms on electrophoresis at pH 6.8.

Protein aggregation is usually maximal at the isoelectric point (Defaye et al., 1994). On subsequent resolubilization these solutions contained the highest concentration of the dimeric metmyoglobin. This was expected since protein-protein interactions would be enhanced. Note that a change of a little more than one pH unit to acid values resulted in no dimer formation on pressure treatment. At alkaline values formation of the dimer still occurred at the pH where no precipitation occurred immediately after pressure treatment (Defaye et al., 1994). This pH dependence suggested that the charge the protein carried was a major factor in determining the amount of dimer formed. Native metmyoglobin has several polar groups with unusual pK values, since they are obscured and are not, in the native protein, accessible to the solvent (Kendrew, 1961). However, on pressure denaturation such groups could ionize and, after being ionized, may not return to unionized forms before the protein refolds. This slight difference in charge distribution may be conducive to two molecules interacting to form a stable dimer if it leads to a slight change in conformation enabling appropriate groups on the surface to interact. With both urea and acid the ionizations either do not take place or the kinetics of refolding do not promote the charges being retained.

The increased concentration of dimer found at higher concentration of metmyoglobin was also expected because of the likelihood of two molecules interacting to form the stable dimer. Such likelihood must increase with increasing concentration. If similar changes occur with other proteins (including enzymes) then the quality of some foods may be modified, immediately after treatment and on subsequent storage.

REFERENCES

Andrews, A.T. 1988. Protein analysis by electrophoresis, recent developments. In *Developments in Food Proteins - 6*, B.J.F. Hudson (Ed.), p. 219–258. Elsevier Applied Sciences, London.

Defaye, A., Ledward, D.A., MacDougall, D.B., and Tester, R.F. 1995. Renaturation of metmyoglobin subjected to high isostatic pressure. Food Chem. (in press).

Gould, G.W. 1994. The microbe as a high-pressure target. In *High-Pressure Treatment of Food*, D.A. Ledward, R.G. Earnshaw, A.P.M. Hastings, and D.E. Johnston (Ed.). Nottingham University Press, Nottingham (in press).

Hite, B.H. 1899. The effect of pressure on the preservation of milk. W. Va. Agric. Exp. Sta. Bull. 58, p. 15–35.

Isaacs, N.S. 1981. *Liquid-Phase High-Pressure Chemistry*, P. 414. John Wiley & Sons, New York.

Kendrew, J.C. 1961. The three-dimensional structure of a protein molecule. Scientific American 205(6): 96–110.

Labbe, R.F., Finch, C.A., Smith, N.J., Doan, R.N., Sood, S.K., and Madan, N. 1979. Erythrocyte protoporphyrin/heme ratio on the assessment of iron status. Clin. Chem. 25: 87–92.

Laemmli, V.K. 1970. Cleavage of structural proteins during the assembly of the heads of bacteriophage T4. Nature 227: 680–685.

Lycometros, C. and Brown, W.D. 1973. Effects of gamma irradiation on myoglobin. J. Food Sci. 38: 971–977.

Morild, E. 1981. The theory of pressure effects on enzymes. In *Advances in Protein Chemistry*, C.B. Afinsen, J.T. Edsoll, and F.M. Richards (Ed.), Vol. 34: 93–116. Academic Press, New York.

Neuhoff, V., Arold, N., Taube, D., and Ehrhardt, N. 1988. Improved staining of proteins in polyacrylamide gels including isoelectric focusing gels with clear background at nanogram sensitivity using Coomassie Brilliant Blue G-250 and R-250. Electrophoresis 9: 255–262.

Sigma Diagnostics 1991. Micro Protein Determination. Procedure No. 690. Sigma Diagnostics, St. Louis, MO.

van den Oord, A.H.A., Wesdorp, J.J., van Dam, A.F., and Verheij, J.A. 1969. Occurrence and nature of equine and bovine myoglobin dimers. Europ. J. Biochem. 10: 140–145.

Zipp, A. and Kauzmann, W. 1973. Pressure denaturation of metmyoglobin. Biochem. 12: 4217–4227.

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Emulsion Stabilization by Maillard-type Covalent Complex of Plasma Protein with Galactomannan

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ABSTRACT

Plasma protein was conjugated with galactomannan (GM) in a controlled dry state at 60°C and 79% relative humidity. The covalent attachment of GM to plasma protein was confirmed by SDS-PAGE and gel chromatography. Emulsifying activity of plasma protein-GM complex was 1.4 times and the emulsion stability of the complex was more than 10 times higher than those of plasma protein. In acidic (pH 3) and salt (0.2M NaCl) systems, the complex showed much more effective emulsifying properties than did commercial emulsifiers. Emulsifying properties of the complex were retained after preheating at 80°C for 30 min. Plasma protein-GM complex prepared by Maillard reaction during heating in a dry state could be used for heat-processed foods as a protein emulsifier.

Key Words: plasma protein, galactomannan, Maillard complex, emulsion, heat stability

INTRODUCTION

PLASMA PROTEIN prepared from animal blood has good emulsifying properties (Tybor et al., 1975; Saito and Taira, 1987). However, such properties are particularly sensitive to heat processing; the emulsifying activity decreased about 60% after treatment at 80°C for 30 min in neutral pH (Saito et al., 1988). The utilization of plasma proteins as food materials is limited because such losses in emulsifying properties and solubility during heat treatments. Improvement of functional properties of plasma proteins would enhance their usefulness in processed foods.

Glycosylation of proteins is an effective way to overcome instability to heating (Marshall and Rabinowitz, 1976; Kitabatake et al., 1985) and to improve functional properties (Marsh et al., 1977; Lee et al., 1979; Kato et al., 1989). Preparation of protein-polysaccharide complexes with excellent emulsifying properties has been accomplished by the attachment of polysaccharides to proteins by a Maillard reaction (Kato et al., 1990; Nakamura et al., 1992; Matsudomi et al., 1994). Accordingly, surface properties of plasma proteins may be improved by conjugation with a polysaccharide using similar techniques. Guar gum is a favorable polysaccharide utilized as a thickener, binder, and stabilizing agent in foods. Its mannase hydrolysate has been developed as a soluble dietary fiber (Yamamoto et al. 1990). Thus, the mannase hydrolysate of guar gum (galactomannan; GM) was applied as a polysaccharide to form complexes with plasma protein.

Our objective was to evaluate the heat stability and emulsifying properties of plasma protein-GM complex formed by Maillard reaction during dry heating.

MATERIALS & METHODS

Materials

A galactomannan preparation (GM, MW 20000-30000) supplied by Taiyo Chemicals Co. (Yokkaichi, Japan) was dialyzed vs deionized water to remove the oligomeric saccharides from the mannase hydrolysate of guar gum and freeze-dried. The commercial emulsifiers, Sunsoft SE-16 (sucrose-fatty acid ester) and Sunsoft Q-18S (decaglycerol monostearate) were supplied by Taiyo Chemicals Co. Sepharose CL-4B was obtained from Pharmacia. All other chemicals were reagent grade.

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Preparation of plasma protein

Whole porcine blood was obtained from Itoh Ham Food Co. (Tokyo, Japan). The blood was collected at the time of slaughter and sodium citrate solution (5% w/v) was added as an anticoagulant at 100 mL/L of blood. The mixture was immediately cooled to 4°C in an ice bath and centrifuged at 6400 × *g* at 4°C for 15 min. The supernatant was dialyzed vs deionized water at 4°C and freeze-dried.

Preparation and dry heating of plasma protein-GM

A mixture of plasma protein-GM (1:1, w/w) was dissolved in distilled water, freeze-dried and incubated at 60°C and 79% relative humidity for 0, 1, 3, 5 or 7 days. This mixing ratio was chosen after a preliminary screening to obtain the best emulsifying properties. For control samples, the same treatment was also applied to plasma protein without GM. The dry-heated samples were preserved in a brown bottle with a self-sealing cap at 4°C.

Determination of protein solubility

The dry-heated samples were dissolved in 0.066M sodium phosphate buffer (pH 7.4) to give 0.2% (w/v) plasma protein concentration. After centrifugation at 5600 × *g* for 10 min at 4°C protein concentration was determined by the modified Lowry et al. (1951) method of Miller (1959). Protein solubility was represented as the ratio to native plasma protein (0 day incubation).

Plasma protein-GM complex

Nonreactive plasma protein and plasma protein polymer in the crude complex were removed by gel filtration on a Sepharose CL-4B column (2 × 80 cm) using 0.066M carbonate buffer (pH 10.0) containing 0.2M NaCl and 0.02% (w/v) sodium azide as eluent. To detect the protein, absorbance was monitored at 280 nm, while GM was detected by the phenol-sulfuric acid method (Dubois et al., 1956). All fractions containing plasma protein-GM complex were collected together, dialyzed vs distilled water, and freeze-dried.

Heat treatment and turbidimetry

Plasma protein samples were dissolved in 0.066 M sodium phosphate buffer (pH 7.4) with or without 0.2M NaCl to give 1% (w/v) plasma protein. The protein solutions (3 mL) in glass vials (Pyrex culture tubes 9826; Corning Glass Co., Corning, NY)) were heated at 80°C up to 30 min with gentle shaking. After heat treatment, the solutions were immediately cooled in a water bath to room temperature, and then the turbidity of the protein suspensions was measured at 600 nm.

Gel electrophoresis

SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was done according to the method of Laemmli (1970) using 10% acrylamide separating gel and 5% acrylamide stacking gel containing 0.1% (w/v) SDS. Samples (20 μL containing 0.2% plasma protein) were prepared in Tris-glycine buffer (pH 8.3) containing 1% (w/v) SDS and 1% (w/v) 2-mercaptoethanol (ME). Electrophoresis was performed at constant current of 10 mA for 5 hr in Tris-glycine buffer containing 0.1% (w/v) SDS. After electrophoresis, gel sheets were stained with Coomassie brilliant blue R-250 for protein and with Fuchsin for carbohydrate.

Table 1—Emulsifying properties of plasma protein incubated with or without galactomannan (GM) at 60°C and 79% relative humidity[a]

Samples	Emulsifying properties	Incubation time (days) 0	1	3	5	7
Plasma-GM mixture	Activity[b]	0.82 ± 0.03	1.08 ± 0.04	1.15 ± 0.03	1.16 ± 0.03	1.15 ± 0.05
	Stability[c]	0.70 ± 0.05	2.00 ± 0.10	3.80 ± 0.10	>10	>10
Plasma alone	Activity[b]	0.83 ± 0.02	0.85 ± 0.03	1.05 ± 0.05	1.01 ± 0.05	0.89 ± 0.07
	Stability[c]	0.70 ± 0.05	0.80 ± 0.05	0.90 ± 0.03	0.80 ± 0.10	0.60 ± 0.10

[a] The emulsifying properties were measured in 0.066 M phosphate buffer, pH 7.4. Data are represented as mean values ± standard deviation of three separate experiments.
[b] Optical density at 500 nm.
[c] In minutes.

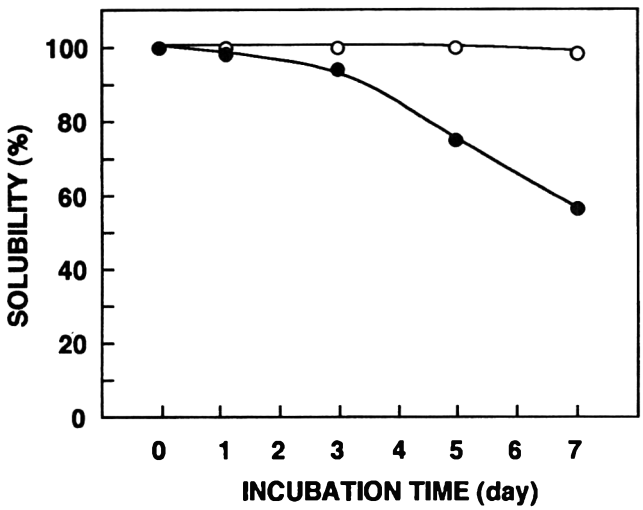

Fig. 1—Solubility of plasma protein incubated with or without galactomannan (GM) at 60°C and 79% relative humidity. The solubility of dry-heated samples is represented as the ratio to native plasma protein (0 day incubation). (○) Plasma protein-GM mixture (1:1, w/w); (●) plasma protein alone.

Emulsifying properties

Emulsifying properties were determined by the turbidimetric procedure of Pearce and Kinsella (1978). Sample solutions containing 0.1% (w/v) plasma protein were prepared with different buffer solutions. Protein solution (3 mL) and 1.0 mL corn oil were homogenized in a glass tube at 25°C for 1 min with an Ultra-Turrax (Hansen Co., Germany) at 12000 rpm. A sample (0.1 mL) of emulsion was taken from the bottom of the glass tube after standing for 0, 1, 2, 3, 5 and 10 min and diluted with 5.0 mL of 0.1% (w/v) SDS solution, and then the turbidity was measured at 500 nm. The emulsifying activity was determined from the turbidity measured immediately after emulsion formation (0 min). Emulsion stability was estimated by measuring the half-life of the decay of emulsion at 500 nm starting immediately after the emulsion had formed. Data were represented as mean values (± standard deviation) of three separate experiments.

RESULTS & DISCUSSION

SOLUBILITY OF PLASMA PROTEIN incubated with or without GM was related to incubation time (Fig. 1). The plasma protein incubated without GM caused a decrease in solubility during incubation for 7 days at 60°C. No detectible changes occurred in solubility of the protein-GM mixture during incubation for 7 days. This indicated that insolubilization of plasma protein

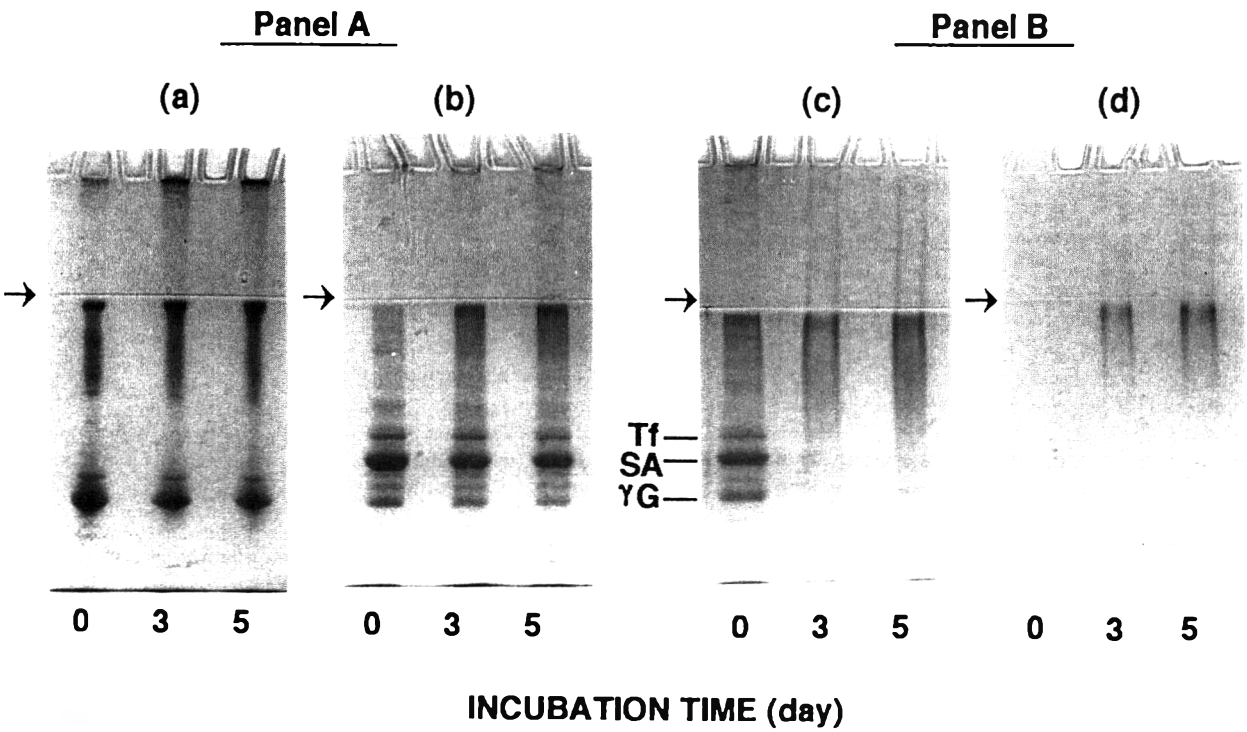

Fig. 2—SDS-polyacrylamide gel electrophoretic patterns of plasma protein incubated with or without galactomannan (GM) at 60°C and 79% relative humidity. Panel A shows protein stain of plasma protein alone in the absence (a) and presence (b) of 2-mercaptoethanol (ME). Panel B shows protein stain (c) and carbohydrate stain (d) of plasma protein-GM mixture (1:1, w/w) in the presence of ME. The arrows show the boundary between stacking and separating gels. Tf, transferrin; SA, serum albumin; γ-globulin (heavy chain).

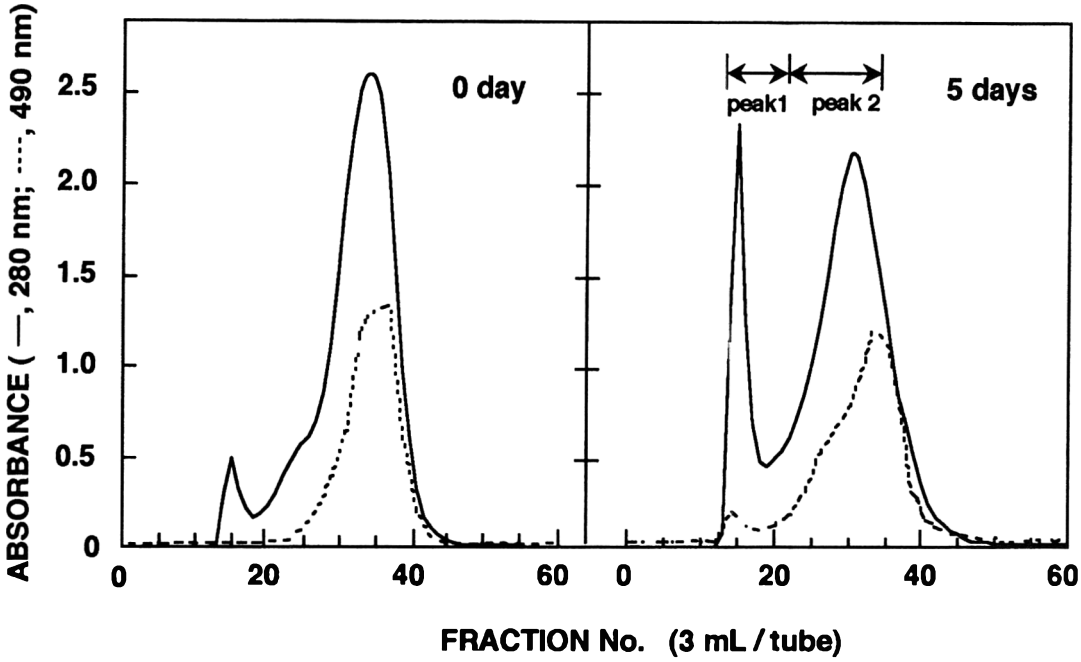

Fig. 3—Gel filtration patterns of plasma protein-galactomannan mixture (1:1, w/w) incubated at 60°C and 79% relative humidity. The fractions (peak 1 and peak 2, indicated by arrows) were used for further experiments as complex 1 and complex 2, respectively. Protein and carbohydrate concentrations were measured from absorbance at 280 nm and 490 nm after color development using phenol-sulfuric acid, respectively.

might have been suppressed by an interaction with GM present during dry heating.

The emulsifying properties of plasma protein incubated with or without GM for different storage times (days) (Table 1) showed the protein-GM mixture incubated for 0 hr was similar to the control (0 hr incubation). This indicates that GM alone had no effect on emulsifying properties of plasma protein. The emulsifying properties of plasma protein incubated without GM increased slightly after 3 days but decreased after 7 day, possibly because of lower solubility of the protein. The emulsifying activity and emulsion stability were enhanced with increases in incubation time at 60°C, reaching a steady state after 5 days. The emulsifying activity was 1.4 times higher and emulsion stability more than 10 times better than control plasma protein-GM mixture (0 hr incubation), suggesting the formation of a plasma protein-GM complex.

Covalent attachment of GM to plasma protein was confirmed by SDS-PAGE after reduction with ME. The SDS-PAGE patterns of plasma protein incubated with or without GM at 60°C and 79% relative humidity were compared for different incubation times (Fig. 2). The plasma protein samples incubated without GM for 3 and 5 days were analyzed by SDS-PAGE (Fig. 2, panel A-a). The appearance of a protein band remained on top of the stacking gel, in the absence of ME and increased with increases in incubation time. The protein was dissociated almost completely into the constituents of plasma protein in the presence of ME (Fig. 2, panel A-b), suggesting that the highly polymerized protein had been formed by disulfide bonds. The plasma-GM mixtures incubated for 3 and 5 days gave a dispersed protein band near the top of the separating gel even in the presence of ME (Fig. 2, panel B-c). The band detected with carbohydrate stain was similar to the dispersed protein band (Fig. 2, panel B-d), indicating the covalent attachment of GM to the protein and the formation of plasma protein-GM complex.

The elution profiles of the plasma protein-GM mixture separated by gel filtration were compared (Fig. 3). The mixture incubated for 5 days revealed 2 protein peaks shifted towards the higher molecular weight fraction. The two peaks overlapped with the carbohydrate peak, indicating that GM was covalently attached to plasma protein. The protein peak which eluted at shorter times seemed to be a highly polymerized complex. The

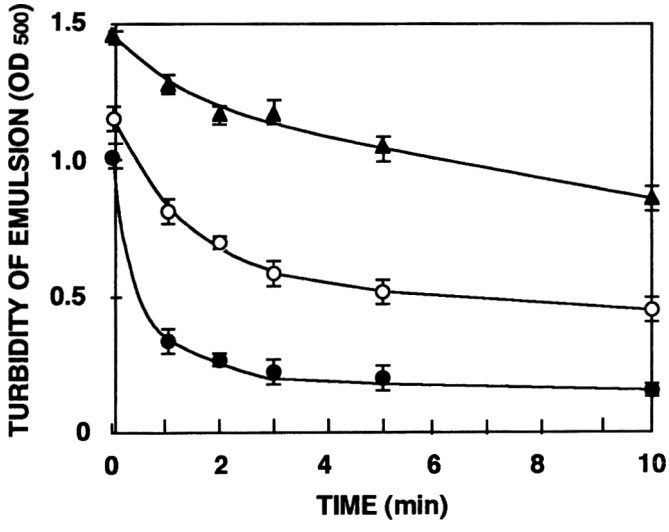

Fig. 4—Emulsifying properties of plasma protein-galactomannan (GM) complexes in 0.066M sodium phosphate buffer (pH 7.4). (●) Plasma protein-GM complex (complex 1); (▲) plasma protein-GM complex (complex 2); (○) plasma protein-GM mixture (1:1, w/w) incubated for 5 days. Each point is the mean ± standard deviation of three separate experiments.

fractions indicated by horizontal arrows (Fig. 3) were pooled, dialyzed, and used for further experiments. The two plasma protein-GM complex fractions (peak 1 and peak 2) obtained from the plasma protein-GM mixture incubated for 5 days were labelled complexes 1 and 2, respectively, in the order of elution time.

The emulsifying properties of complexes 1 and 2 were compared with plasma protein-GM mixture incubated for 5 days (Fig. 4). Complex 2 had higher emulsifying properties in a neutral buffer system (0.066M sodium phosphate buffer, pH 7.4), while the emulsifying properties of complex 1 decreased considerably more than those of the plasma protein-GM mixture. The difference in emulsifying properties between the two com-

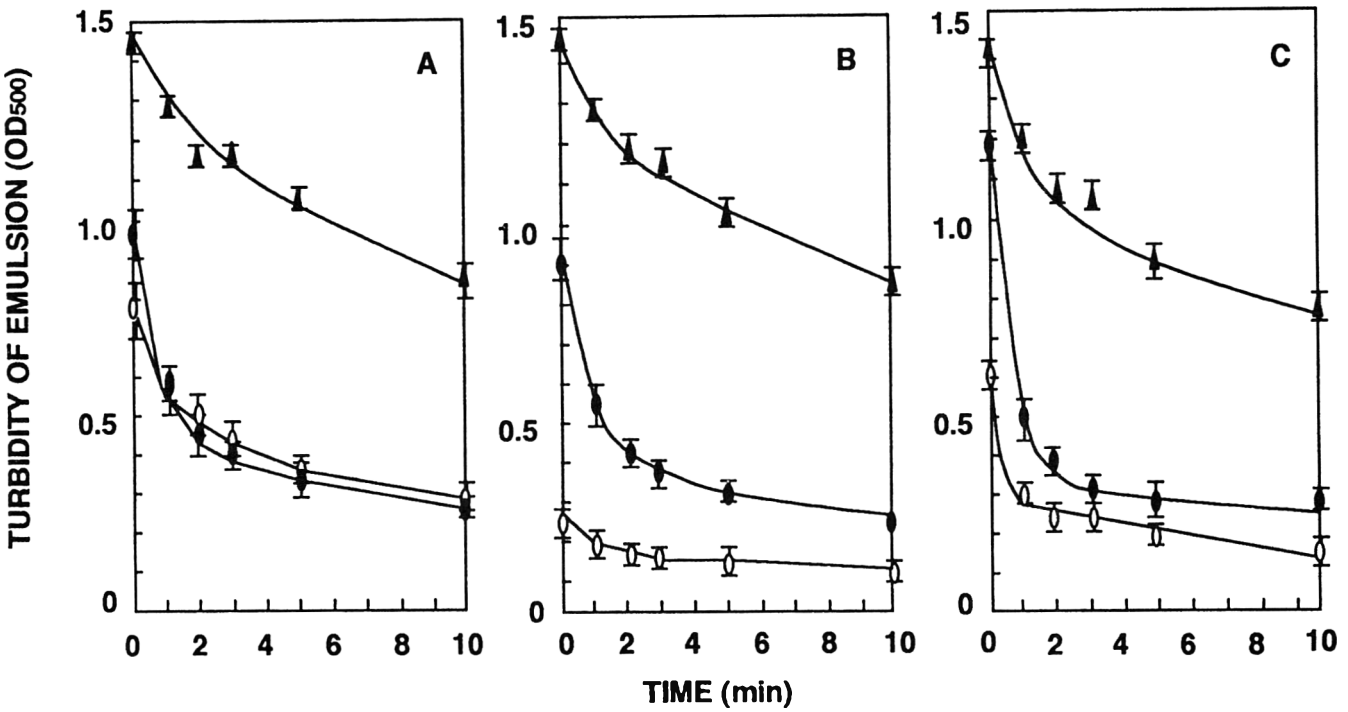

Fig. 5—Emulsifying properties of plasma protein-galactomannan (GM) complex and commercial emulsifiers. (▲) Plasma protein-GM complex (complex 2); (○) commercial emulsifier, Sunsoft SE-16, sucrose-fatty acd ester; (●) commercial emulsifier, Sunsoft Q-18S, decaglycerol monostearate. (A) In 0.066 M sodium phosphate buffer (pH 7.4); (B) in 0.066 M sodium citrate buffer (pH 3.0); (C) in 0.066 M sodium phosphate buffer (pH 7.4) containing 0.2M NaCl. Each point is the mean ± standard deviation of three separate experiments.

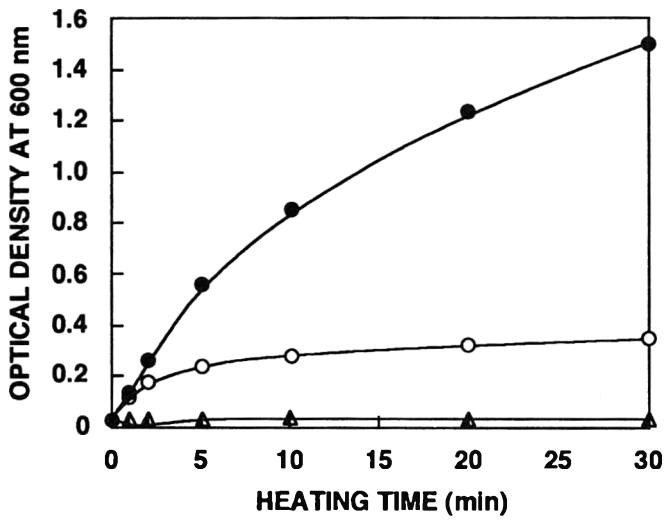

Fig. 6—Changes in turbidity of plasma protein-galactomannan (GM) complex and native plasma protein during heat treatment. Protein solution (1%, w/v) was heated at 80°C in 0.066M sodium phosphate buffer (pH 7.4) with (closed symbols) or without (open symbols) 0.2M NaCl. (●, ○) Native plasma protein; (▲, △) plasma protein-GM complex (complex 2).

plexes may result from steric hindrance from the polymerization of protein.

The emulsifying properties of complex 2 were compared with those of commercial emulsifiers, Sunsoft™ SE-16 and Sunsoft™ Q-18S in various conditions (Fig. 5). Complex 2 had much better emulsifying properties than the commercial emulsifiers in the neutral buffer system (Fig. 5A). The emulsifying properties of complex 2 were much higher than those of the commercial emulsifiers in an acidic buffer system (0.066M citrate buffer, pH 3.0) (Fig. 5B). In addition, complex 2 retained higher emulsifying properties even in a high-salt buffer system (0.066M sodium phosphate buffer, pH 7.4, containing 0.2 M NaCl). However, the emulsifying activity of complex 2 in the high-salt buffer was slightly lower than in the other buffer systems (Fig. 5C). Such excellent emulsifying properties of plasma protein-GM complex (complex 2) in both acidic and high-salt conditions could be advantageous for applications in processed foods.

Table 2—Emulsifying properties of a plasma protein-glactomannan (GM) complex and plasma protein after heat treatment[a]

Samples	Emulsifying properties	Heat treatment (30 min at 80°C) Before	After
Plasma-GM complex	Activity[b]	1.42 ± 0.04	1.40 ± 0.03
	Stability[c]	>10	>10
Plasma alone	Activity[b]	0.82 ± 0.02	0.60 ± 0.07
	Stability[c]	0.70 ± 0.05	0.40 ± 0.07

[a] The emulsifying properties were measured after heat treatment for 30 min at 80°C in 0.066 M phosphate buffer (pH 7.4) containing 0.2 M NaCl. The plasma protein-GM complex is peak 2 fraction (complex 2) obtained from plasma protein-GM mixture incubated for 5 days. Data are represented as mean values ± standard deviation of three separate experiments.
[b] Optical density at 500 nm.
[c] In minutes.

In order to examine heat stability, coagulation during heat treatment was monitored by turbidity at 600 nm. The changes in turbidity of 1% (w/v) plasma protein sample solution (in 0.066M phosphate buffer, pH 7.4) during heat treatment at 80°C for 30 min were compared (Fig. 6). The turbidity of native plasma protein showed slight increases during heat treatment without 0.2M NaCl, while the turbidity increased rapidly during heating in the presence of 0.2 M NaCl. Shimada and Matsushita (1981) reported that serum albumin which is the main component of bovine plasma protein showed a rapid heat coagulation in the presence of NaCl at a neutral pH. The same effect of NaCl on heat coagulation was also observed with porcine plasma protein in our results. On the other hand, the turbidity of the plasma protein-GM complex (complex 2) was very low during heat treatment for 30 min and remained at a low level even in the presence of NaCl. This result indicated that coagu-

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Biochemical and Functional Properties of Myofibrils from Pre- and Post-Spawned Hake (*Merluccius hubbsi Marini*) Stored on Ice

S.I. ROURA and M. CRUPKIN

ABSTRACT

Enzymatic activities assayed at beginning of storage in myofibrils from post-spawned hake were 3× those in myofibrils from pre-spawned hake. Ca^{2+} sensitivity of myofibrils from pre-spawned hake was 40% less than that of myofibrils from post-spawned hake. The profiles of SDS-PAGE gels of pre-spawned myofibrils at beginning of storage showed a partially denatured myosin heavy chain, and polypeptide bands under myosin heavy chain. They probably represent proteolytic fragments procuced by degradation of MHC *in vivo*. No proteolysis was detected in myofibrils during storage. These results help predict functional properties of fish proteins and changes during storage.

Key Words: hake, myofibrils, pre- and post-spawning, enzymes

INTRODUCTION

CHANGES IN THE ACTOMYOSIN COMPOSITION of mature Argentinian hake (*Merluccius hubbsi Marini*) are influenced by the metabolic state of the fish and related to its reproductive cycle (Crupkin et al., 1988). Crupkin et al. (1988) reported that, when compared with actomyosin from resting and post-spawning periods, the actomyosin from pre-spawned fish had significantly lower relative percentages of myosin. Changes in biochemical, physicochemical and functional properties of hake actomyosin during the reproductive cycle of the fish reflect changes previously reported in the actomyosin complex (Beas et al., 1988; Montecchia et al., 1990; Roura et al., 1990). Actomyosin from fish caught in post-spawning condition had higher ATPase activities, reduced viscosity and better gelling properties than those of pre-spawning stages (Beas et al., 1988; Roura et al., 1990). Moreover, a loss of the filamentous structure in actomyosin from pre-spawned hake related to a decrease in the affinity between myosin and actin has been reported (Roura et al., 1992a).

Functional and textural characteristics of meats depend mainly on myofibrillar proteins (Goll et al., 1977). This is more important for fish muscle than for mammalian muscle because of collagen content (Brown, 1986). The study of functional properties of fish myofibrillar proteins is important for determining and predicting the final quality of fishery products. Much is known about biochemical, physicochemical and functional properties of actomyosin from iced fish, related to the metabolic state and gonadal maturation of fish (Crupkin et al., 1988; Montecchia et al., 1990; Roura et al., 1990). However, information is scarce concerning the influence of gonadal conditions on the properties of myofibrils purified from hake during ice storage. Myofibrils are a highly organized muscle structure similar to whole muscle and they are useful for the study of the contractile proteins of skeletal muscle (Yasui et al., 1975). Myofibrils are responsible for contraction in the living cell and for most of the desirable qualities of muscle used as food (Robson et al., 1991).

The changes in actomyosin related to the gonadal condition of hake have been explained by the hypothesis that myosin is more sensitive than actin to proteolysis *in vivo* (Crupkin et al., 1988). However, actomyosin is a macromolecular complex in which myofibrillar proteins do not retain the integrity of muscle because of the high ionic strength of the purification solution. Myofibrils are important for the study of biochemical and functional properties because they are somewhat related to both whole muscle and purified proteins. Our objective was to study the biochemical and functional properties of myofibrils from pre- and post-spawned hake stored on ice to help determine why myofibrils retain much of the structural organization of whole muscle.

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MATERIALS & METHODS

Fish source

Hake (*Merluccius hubbsi*) were harvested by commercial vessels in the Southeast Atlantic Ocean between 36° and 53° S. latitude from October 1990 to May 1993. Fish were kept on ice until they reached the laboratory in an early post-rigor condition. All fish were 35–45 ± 0.1 cm long (from snout to tip of mid-caudal ray). Fish were stored in boxes and covered with ice. At days 0, 3, 7 and 10 of storage, six females in pre-spawning condition and six females in post-spawning condition were removed for analysis. The gonadal condition was determined using histological techniques described by Goldemberg et al. (1987).

Preparation of myofibrils

Myofibrils were prepared as described by Yasui et al. (1975) as previously reported (Roura et al., 1992b). However, Triton X-100 was not used for further purification of hake myofibrils because it inactivates contractile proteins (Roura et al., 1992b; Roura et al., 1993).

Protein determination

Protein concentration was determined on aliquots of the extracts by the Lowry method (Lowry et al. 1951).

Myofibrillar ATPase activity

Enzymatic activity of myofibrils was measured at 37°C in a 30 mM Tris-maleate buffer (pH 7.0). Specific conditions for each enzyme were 0.12 mg.mL^{-1} of protein, 0.75 mM ATP, 60 mM KCl and 10 mM $CaCl_2$ for the Ca^{2+}-ATPase activity; 0.25 mg.mL^{-1} of protein, 0.75 mM ATP, 60 mM KCl, 2 mM $MgCl_2$ and 0.5 mM of ethyleneglycol-bis-(beta-aminoethyl ether)N,N'-tetraacetic acid (EGTA) for the Mg^{2+}-(EGTA)-ATPase activity 0.12 mg.mL^{-1} of protein, 0.75 mM ATP, 60 mM KCl, 0.1 mM $CaCl_2$ and 2 mM $MgCl_2$ for the Mg^{2+}-Ca^{2+}-ATPase activity; and 0.25 m.mL^{-1} of protein, 0.75 mM ATP, 60 mM KCl and 2 mM $MgCl_2$ for the Mg^{2+}-ATPase activity.

A final incubation volume of 3 mL was used in all cases. Incubation times in minutes were for Ca^{2+}, 5; Mg^{2+}, 1; Mg^{2+}-(EGTA), 5; and Mg^{2+}-Ca^{2+}-ATPases, 4. Reactions were interrupted by addition of 1 mL cold 40% trichloroacetic acid solution. Liberated phosphorus was determined according to the method of Chen et al. (1956).

Densitometric analysis of myofibril patterns in SDS-PAGE

SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out in 10% gels according to the procedure of Portzio and Pearson (1977) using a Shandon vertical apparatus. Areas of bands were determined quantitatively, by scanning gels at 600 nm with a Beckman DU-8 UV-

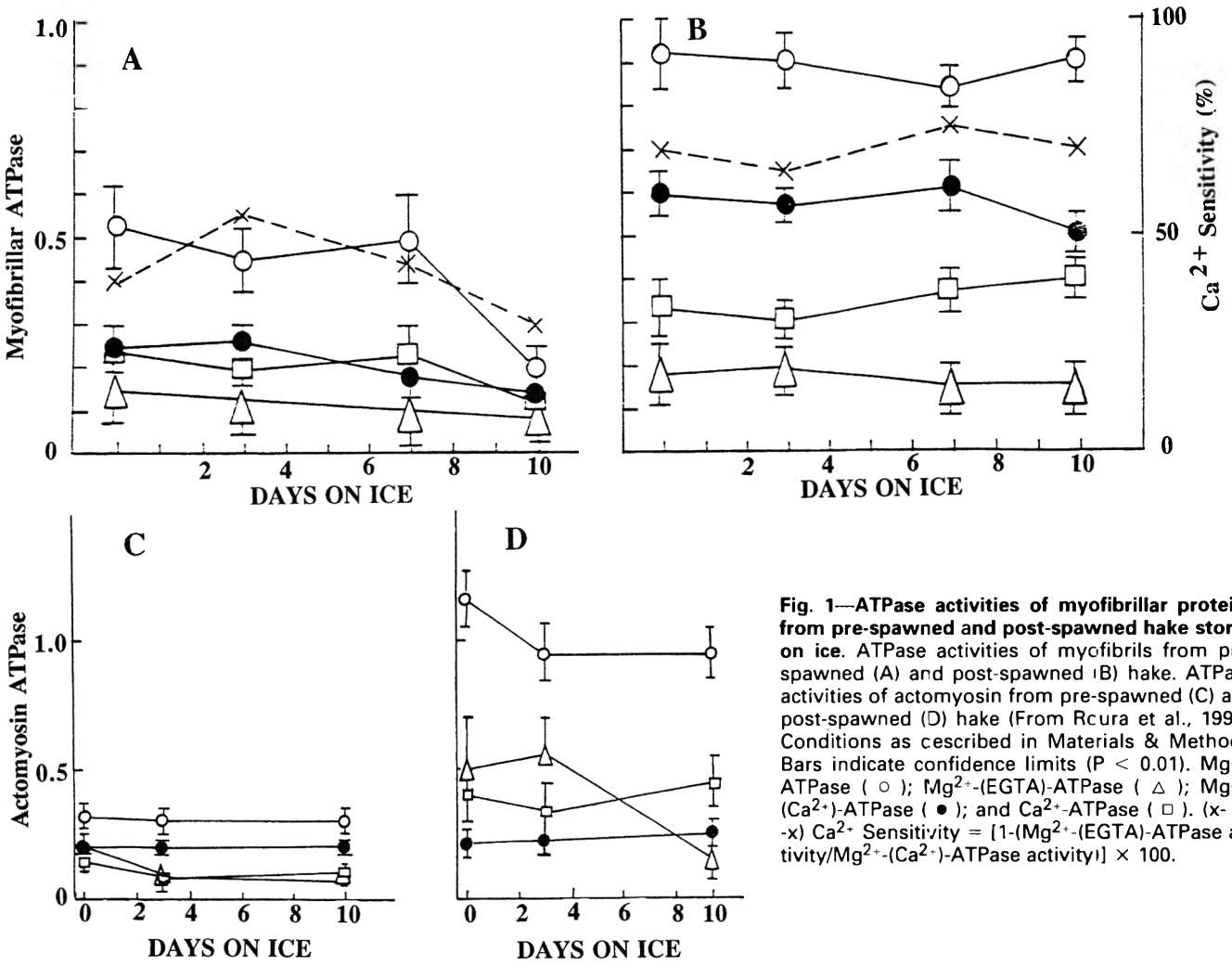

Fig. 1—ATPase activities of myofibrillar proteins from pre-spawned and post-spawned hake stored on ice. ATPase activities of myofibrils from pre-spawned (A) and post-spawned (B) hake. ATPase activities of actomyosin from pre-spawned (C) and post-spawned (D) hake (From Roura et al., 1990). Conditions as described in Materials & Methods. Bars indicate confidence limits ($P < 0.01$). Mg^{2+}-ATPase (○); Mg^{2+}-(EGTA)-ATPase (△); Mg^{2+}-(Ca^{2+})-ATPase (●); and Ca^{2+}-ATPase (□). (x- - -x) Ca^{2+} Sensitivity = [1-(Mg^{2+}-(EGTA)-ATPase activity/Mg^{2+}-(Ca^{2+})-ATPase activity)] × 100.

spectrophotometer equipped with a DU-8 gel scanning system. The amount of protein loaded on the gel was 30 µg.

Statistical analysis

The statistical significance of differences between mean values was determined by analysis of variance, using Duncan's new multiple range test (Steel and Torrie, 1960).

RESULTS & DISCUSSION

ENZYMATIC ACTIVITIES and Ca^{2+} sensitivity of myofibrils from pre- and post-spawned hake (Fig. 1A,B) showed Mg^{2+}-, Mg^{2+}-(Ca^{2+})-, and Mg^{2+}-(EGTA)-ATPase activities were respectively related to actin/myosin interaction in the presence of endogenous Ca ions, exogenous Ca ions, and absence of Ca ions (Roura et al., 1990). Ca^{2+}-ATPase activity is a good indicator of myosin functionality in contractile proteins. At beginning of storage, the Mg^{2+}-, Mg^{2+}-(Ca^{2+})-, and Ca^{2+}-ATPase activities of myofibrils were higher ($P < 0.01$) in post-spawned than in pre-spawned fish (Fig. 1A,B). Similar values of Mg^{2+}-(EGTA)-ATPase activity were obtained for both gonadal stages. As a consequence the Ca^{2+} sensitivity was 40% lower in myofibrils from pre-spawned hake than in those isolated from post-spawned hake (Fig. 1A,B).

Ca^{2+} sensitivity is a good indicator of the Ca^{2+} regulation of myofibrillar proteins. No significant changes were observed in Ca^{2+} sensitivity of pre- and post-spawned myofibrils during ice storage ($P < 0.01$). However, a higher percentage of initial Ca^{2+} sensitivity (80%) was found in myofibrils from post-spawned hake (Fig. 1 A,B). The Ca^{2+} sensitivity of myofibrillar proteins was attributed to the activity of native tropomyosin (Ebashi et al., 1968). The troponin-tropomyosin complex is necessary for control by Ca^{2+} ions of the actin-myosin interaction in vertebrate striated muscles (Huxley, 1972). The loss of Ca^{2+} sensitivity is considered to be due to the filamentation of myofibrils caused by the hydrolysis of proteases (Tokiwa and Matsumiya, 1969).

According to studies of Seki and Hasegawa (1978), Shimomura and Seki (1978), and Seki et al. (1979), the loss of Ca^{2+} sensitivity of myofibrillar proteins during ice storage was not due to hydrolysis of tropomyosin and troponins by protease. It was caused by modification of actin-myosin interactions by oxidation of thiol groups of the myosin moiety. Mg^{2+}-ATPase is responsible for interaction between actin and myosin in the presence of endogenous Ca (Roura et al., 1990). The activity of this enzyme was 40% lower in myofibrils purified from pre-spawned hake (Fig. 1A,B). Resulting modifications in actin-myosin interactions could cause loss of Ca^{2+} sensitivity.

Konagaya (1982) reported enhanced protease activity in muscle of chum salmon (*Oncorhyncus keta*) during spawning migration attributed to cathepsins activity. Matsumiya et al. (1991) reported that the acid, neutral and alkaline proteinase activities in the muscle of common mackerel increased remarkably in the spawning season and this was related to development of the gonads. Martone et al. (1991) reported that hake skeletal muscle contained a trypsin-like serine protease which might be involved in the catabolism of myofibrillar proteins *in vivo*. Low myosin/actin ratios were observed in actomyosin from pre-spawned hake (Crupkin et al., 1988). The presence of less myosin in this

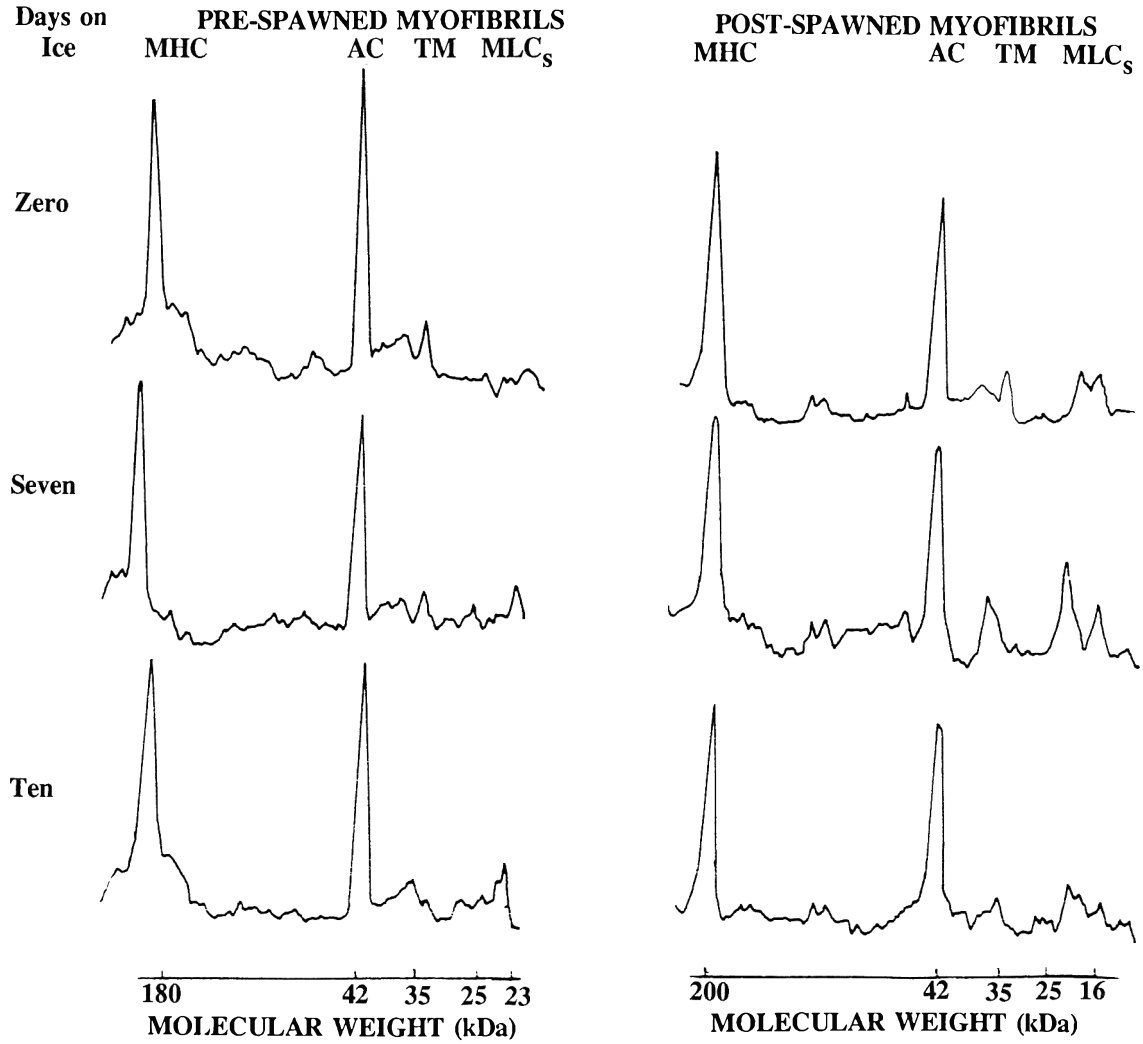

Fig. 2—Densitometric analysis of myofibril profiles in SDS-PAGE (10%) from pre- and post-spawned hake stored on ice. Molecular weights, in daltons, are indicated. MHC = myosin heavy chain; A = actin; TM = tropomyosin; MLC_S = myosin light chains.

phase of the gonadal cycle was explained by the hypothesis that myosin was more sensitive to proteolysis than actin *in vivo* (Crupkin et al., 1988). Therefore, loss of Ca^{2+} sensitivity in myofibrils, from pre-spawned fish could be related to an increment in proteinase activity which selectively degrades myosin during gonadal maturity. However, from our results, the possibility that it is due to oxidation of thiol groups of the myosin moiety should still be considered.

No changes ($P < 0.01$) in the enzymatic activities of myofibrils from post-spawned hake were observed during ice storage (Fig. 1B). The enzymatic profiles for pre-spawned myofibrils showed that the Mg^{2+}-ATPase retained the activity after 7 days of ice storage (Fig. 1A), while enzymatic activities of the others gradually decreased during ice storage.

The inactivation rate of myofibrillar ATPase was lower than that of isolated actomyosin ATPase (Fig. 1C,D). Ionic strength influences protein-to-protein interactions and protein solubilities. High ionic strength decreases actin-myosin interactions in the relaxed, activated, or rigor states of muscle (Wu and Smith, 1987). The aggregation of myosin molecules, the actin-tropomyosin complex and other myofibrillar proteins into myofibrils involves electrostatic interactions (Wu and Smith, 1987) which are affected by ionic strength. Increasing ionic strength decreases the sphere of each charge on the proteins (Raymond and Zubay, 1984), and this can weaken the structural integrity of myofibrils.

The activity of Mg^{2+}-ATPase, the enzyme responsible for actin-myosin interactions, was 65% higher in hake myofibrils than in hake actomyosin in the pre-spawned period (Fig. 1A,B). Seki and Narita (1980) found similar results in myofibrils and actomyosin isolated from carp muscle. Differences in ATPase activities in myofibrils and actomyosin could result from differences in ionic strengths used in purification methods: low ionic strength for myofibrils and high ionic strength for actomyosin.

At beginning of storage EGTA produced 71 and 80% inhibition of myofibrillar Mg^{2+}-ATPase in pre- and post-spawned hake, respectively (Fig. 1A,B). The EGTA inhibition of actomyosin ATPase was 37 and 56% for pre- and post-spawned hake, respectively (Fig. 1C,D). Myofibrils, an organized muscle structure, retained the troponin-tropomyosin structure in a native form. This could be the reason for the higher EGTA inhibition of Mg^{2+}-ATPase observed in myofibrils as compared with isolated actomyosin.

Profiles of SDS-PAGE gels of myofibrils from pre- and post-spawned hake during ice storage (Fig. 2) indicate at zero time-pre-spawned myofibrils showed the presence of a partially degraded myosin heavy chain, with molecular weight 180 kDa. Polypeptide bands under the myosin heavy chain were also present, probably representing proteolytic fragments produced by degradation of the myosin heavy chain *in vivo* (Fig. 2). SDS-PAGE patterns of post-spawned myofibrils showed characteristic polypeptide bands of myofibrils with molecular weights corresponding to native contractile proteins: myosin heavy chain (MHC), 200 kDa; actin (A), 42 kDa; tropomyosin (TM), 35 kDa and myosin light chains (MLCS), 21–23 kDa (Fig. 2). These results confirmed those reported for actomyosin profiles of pre- and post-spawned hake (Roura et al., 1990). In that report, the SDS-PAGE pattern of actomyosin from pre-spawned hake

showed an absence of heavy myosin chain and the presence of a 160 kDa component at zero time. The researchers hypothesized that the 160 kDa component resulted from drastic *in vivo* degradation of heavy myosin due to extreme starvation of the fish (Roura et al., 1990). Matsukura et al. (1981) reported that cathepsin L could degrade myosin and myofibrils *in vitro*, producing a fragment of 160 kDa. An increase of acid hydrolase activity in skeletal muscle after physical stress was also reported (Vihko and Salminen, 1983). The protease activity enhancement in salmon muscle during spawning migration (possibly cathepsins type) was related to expenditure of body protein for energy required to make the long trip and concurrently to develop mature gonads (Konagaya, 1982). A similar situation could occur during gonadal development of hake. Therefore, for the gonadal growth period, the presence of a partially degraded myosin heavy chain, both in myofibrils and actomyosin, could be explained by an increased proteinase activity in muscle.

No proteolysis was detected in characteristic polypeptide bands of major myofibrillar proteins during ice storage of pre- or post-spawned hake (Fig. 2). These results confirmed those reported for actomyosin purified from hake stored on ice (Roura et al., 1990). Results indicate that changes in hake myofibrils were related to gonadal condition and reflect changes in actomyosin. Myofibrils retain much of the structural organization of muscle. Thus, seasonal changes found in the biochemical and functional properties of hake myofibrils probably result from changes that occur in the muscle *in vivo*.

CONCLUSION

MYOFIBRILS from post-spawned hake have more active biochemical and functional properties than those from pre-spawned fish. Enzymatic activities at zero time from post-spawned hake were 3× those from pre-spawned hake. Ca^{2+} sensitivity of myofibrils from pre-spawned hake was 40% lower than that of myofibrils from post-spawned hake. The biological condition related to the reproductive cycle influenced biochemical and functional properties. Myofibrils from pre-spawned hake showed the presence of a partially denatured myosin heavy chain, related to an increase of proteolytic activity which selectively degrades heavy myosin chain. No further degradation of major components of myofibrils occurred after 10 days of ice storage in fish caught before or after spawning.

REFERENCES

Beas, V.E., Crupkin, M., and Trucco, R.E. 1988. Gelling properties of actomyosin from pre- and post-spawning hake (Merluccius hubbsi). J. Food Sci. 53: 1322–1326.

Brown, W.D. 1986. Fish muscle as food. Ch. 13, in *Muscle as Food*, P.J. Bechtel (Ed.). Academic Press, Inc., Orlando, FL.

Chen, P.S., Toribara, T.Y., and Warner, H. 1956. Microdeterminations of phosphorus. Analyt. Chem. 28: 1756–1758.

Crupkin, M., Montecchia, C.L., and Trucco, R.E. 1988. Seasonal variations in gonadosomatic index, liver somatic index and myosin/actin ratio in actomyosin of mature hake (*Merluccius hubbsi*). Comp. Biochem. Physiol. 89A: 7–10.

Ebashi, S., Kodama, A., and Ebashi, F. 1968. Troponin. I. Preparation and physiological function. J. Biochem. 65: 465.

Goldemberg, A.L., Paron, L., and Crupkin, M. 1987. Acid phosphatase activity in pre- and post-spawning hake (Merluccius hubbsi). Comp. Biochem. Physiol. 87A: 845–849.

Goll, D.E., Robson, R.M., and Stromer, M.H. 1977. Muscle proteins. Ch. 6, in *Food Proteins*, J.R. Whitaker and S.R. Tannenbaum (Ed.). AVI Publishing Co. Westport, CT.

Huxley, H.E. 1972. Molecular basis of contraction in cross-striated muscles. V1, in *The Structure and Function of Muscle*, G.H. Bourne (Ed.). Academic Press, Inc., New York.

Konagaya, S. 1982. Enhanced protease activity in muscle of chum salmon (*Oncorhyncus keta*) during spawning migration. Bull. Japan. Soc. Sci. Fish. 48(10): 1503.

Lowry, O.H., Rosebrough, N.J., Farr, A.L., and Randall, R.J. 1951. Protein measurement with the folin phenol reagent. J. Biol. Chem. 193: 265–275.

Martone, C.B., Busconi, L., Folco, E., and Sanchez, J.J. 1991. Detection of a trypsin-like serine protease and its endogenous inhibitor in hake skeletal muscle. Arch. Biochem. Biophys. 289: 1–5.

Matsukura, U., Okitani, A., Nishimuro, T., and Kato, H. 1981. Mode of degradation of myofibrillar proteins by an endogenous protease Cathepsin L. Biochim. Biophys. Acta 662: 41–47.

Matsumiya, M., Mochizuki, A., and Otake, S. 1991. Seasonal variation of alkaline proteinase activity in the ordinary muscle of common mackerel. Nippon Suisan Gakkaishi 57: 1411.

Montecchia, C.L., Crupkin, M., and Trucco, R.E. 1990. Seasonal variations in biochemical and physicochemical properties of actomyosin and energy content of the liver, gonads and muscle of mature Argentinian hake, *Merluccius hubbsi Marini*. J. Fish Biology 37: 837–843.

Portzio, M. and Pearson, A. 1977. Improved resolution of myofibrillar proteins with sodium dodecyl sulfate polyacrylamide gel electrophoresis. Biochim. Biophys. Acta 490: 27–34.

Raymond, S.F. and Zubay, G. 1984. Analytic techniques of protein. In *Biochemistry*, G. Zubay (Ed.), p. 39. Addison-Wesley Pub. Co. Inc., Reading, PA.

Robson, R.M., Huiatt, T.W., and Parrish, F.C. (Jr). 1991. Biochemical and structural properties of titin, nebulin and intermediate filaments in muscle. Reciprocal Meat Conference Proceedings, Vol. 44: 1–5.

Roura, S.I., Montecchia, C., Goldemberg, A.L., Trucco, R.E., and Crupkin, M. 1990. Biochemical and physicochemical properties of actomyosin from pre- and post-spawned hake (*Merluccius hubbsi*) stored on ice. J. Food Sci. 55: 688–692.

Roura, S.I., Pecci Saavedra, J., Trucco, R.E., and Crupkin, M. 1992a. Electron microscopical and biochemical studies of actomyosin from pre- and post-spawned hake. Comp. Biochem. Physiol. 101B (3): 361–365.

Roura, S.I., Goldemberg, A.L., Trucco, R.E., and Crupkin, M. 1992b. Action of Triton X-100 on the biochemical and functional properties of hake (*Merluccius hubbsi*) myofibrils. Comp. Biochem. Physiol. 102B: 923–927.

Roura, S.I., Roldan, H., Goldemberg, A.L., Trucco, R.E., and Crupkin, M. 1993. Effect of Triton X-100 on the lipid composition, biochemical and functional properties of hake (*Merluccius hubbsi*) myofibrils purified from pre- and post-spawned fish. Comp. Biochem. Physiol. 106B: 191–197.

Seki, N. and Hasegawa, E. 1978. Comparative studies on fish tropomyosins. Bull. Japan. Soc. Sci. Fish. 44: 71.

Seki, N. and Narita, N. 1980. Changes in ATPase activities and other properties of carp myofibrillar proteins during ice storage. Bull. Japan. Soc. Sci. Fish. 46: 207–213.

Seki, N., Ikeda, M., and Narita, N. 1979. Changes in ATPase activities of carp myofibril during ice-storage. Bull. Japan. Soc. Sci. Fish. 45: 791.

Shimomura, M. and Seki, N. 1978. Separation and properties of the troponin components from carp muscle. Bull. Japan. Soc. Sci. Fish. 44: 231–238.

Steel, R.D.G. and Torrie, J.J. 1960. *Principles and Procedure of Statistics*. MacGraw-Hill Book Co., Inc., New York.

Tokiwa, T. and Matsumiya, J. 1969. Fragmentation of fish myofibril. Effect of storage condition and muscle cathepsins. Bull. Japan. Soc. Sci. Fish. 35: 1099.

Vihko, V. and Salminen, A. 1983. Acid hydrolase activity in tissues of mice after physical stress. Comp. Biochem. Physiol. 76B: 341.

Yasui, T., Sumita, T., and Tsunogae, S. 1975. Stability of myofibrillar EDTA-ATPase in rabbit psoas fiber bundles. J. Agric. Food Chem. 6: 1163–1168.

Wu, F.Y. and Smith, S.B. 1987. Ionic strength and myofibrillar protein solubilization. J. Anim. Sci. 65: 597–608.

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Storage Quality of Fresh and Frozen-thawed Fish in Ice

HANNES MAGNÚSSON and EMILÍA MARTINSDÓTTIR

ABSTRACT

The objective was to determine whether traditional quality indexes of fresh (unfrozen) fish like sensory analysis, bacterial counts and trimethylamine content could be applied to thawed whole cod, cod fillets and ocean perch fillets kept in ice. Freezing and short-term freezer storage (≤5 wk at −25°C) had very little effect on bacterial counts. During long-term freezer storage (≥14 wk at −25°C) total counts were reduced as well as counts of trimethylamine oxide-reducing bacteria in cod fillets but not in ocean perch fillets. When the thawed fish was unacceptable the trimethylamine was <1 mgN/100g. Trimethylamine as a spoilage indicator was of no value when evaluating spoilage of thawed whole cod, cod fillets and ocean perch fillets kept in ice.

Key Words: fresh fish, thawed fish, cod, ocean perch, trimethylamine

INTRODUCTION

FROZEN-THAWED FISH, especially fillets are commonly marketed chilled. Such frozen-thawed fish may be kept for varying times in ice. Two very important species in this respect are cod (*Gadus morhua*) and ocean perch (*Sebastes marinus/S. mentella*)

Trimethylamine oxide (TMAO) is generally present in seawater fish. Trimethylamine (TMA) is formed from TMAO by bacterial reduction during iced storage. However during frozen storage TMAO can in gadoid fish species like cod be broken down to dimethylamine (DMA) and formaldehyde (FA) by endogenous enzymes (Hebard et al., 1982). This reaction is very temperature-dependent; the enzyme activity being inhibited if storage temperature is near −29°C (Castell et al., 1974). Relatively few reports have been published on the storage quality of thawed fish in comparison to fresh (unfrozen) fish in ice. Experiments done by Luijpen (1958) on cod fillets with skin kept at different temperatures showed that TMA was produced slower in thawed fillets than in unfrozen ones. Those results were not in accordance with sensory analysis and not explained by differences in bacterial counts. No information was found regarding this difference in TMA formation in whole fish.

Our main objective was to determine whether a difference in TMA formation occurred in thawed cod, kept whole or as fillets in comparison to unfrozen cod, and if so to find the reason. In addition, fillets of ocean perch were examined. Another objective was to examine the effect of freezing and freezer storage on microbiological flora and TMAO. We also evaluated whether methods for assessing spoilage and keeping quality of unfrozen fish like sensory analysis, bacterial counts and chemical indexes (TMA and total volatile bases, TVB) could be applied to thawed fish kept in ice.

MATERIAL & METHODS

Whole cod

On board a trawler cod was bled, gutted and iced into 90 L boxes. Four days from catch some of the cod were frozen whole in a plate freezer and kept at −25°C for 8 wk. At the same time the rest of the cod were iced for a storage trial at 0 to 1°C. The frozen cod was thawed at 15°C and iced in boxes as soon as the core temperature reached 0°C. Both unfrozen and thawed cod were kept iced for ≈3 wk. On each day of sampling, four cods were individually examined.

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Cod fillets in ≈2.25 kg cartons

When examining the effect of freezing and freezer storage on microorganisms and TMAO, fully trimmed cod fillets in 2.25 kg cartons, each containing six units in a plastic envelope, were collected at a freezing plant. Unfrozen cod were examined microbiologically at exactly the same time as comparable cod was placed in a plate freezer. Frozen cartons were kept at −25°C for 18, 23 and 50 wk in three individual experiments. In all experiments samples were also examined after 1 day in freezer. Frozen units were kept for 1 hr at room temperature prior to examination. Each of the six units in the ≈2.25 kg cartons were examined individually. Cod samples from this experiment were not stored in ice after thawing.

Cod fillets

For ice storage experiments on cod fillets, fresh longline cod were used. The cod were bled onboard and gutted ashore. The cod were filleted and skinned within 24 hr from time of catch. Some fillets were iced in boxes while others were tunnel-frozen at the same time. Frozen fillets were kept at −25°C for 1 day, and for 5, 14, 27 and 52 wk. These fillets were thawed at 15°C until core temperature reached 0°C, and then iced immediately and kept as unfrozen fillets at 0 to 1°C for up to 3 wk. Samples for initial bacterial analysis were examined within 1 hr after removal from freezer. During iced storage, three separate fillets were taken on every sampling day for bacteria and chemical studies and an additional three for sensory evaluation.

Ocean perch fillets

Ocean perch were obtained from a local trawler. The ocean perch were kept unbled and ungutted in ice. On arrival on shore, one day from catch, the fish were filleted and skinned. Some fillets were iced in boxes while others were tunnel-frozen at the same time. Frozen fillets were kept at −25°C for 1 day, 7 wk and 25 wk. Fillets were thawed at 15°C until core temperature reached 0°C, then iced immediately and kept as unfrozen fillets at 0 to 1°C up to 3 wk. Samples for initial bacterial analysis were examined within 1 hr after removal from freezer. During ice storage three separate samples were taken on every sampling day for bacterial and chemical studies and an additional three for sensory evaluation. Each sample consisted of three fillets.

Bacterial counts

When examining whole cod, 3 × 7.5 cm^2 skin samples were cut along the lateral line (behind gills, mid region, tail region) and placed in a stomacher bag containing 60 mL Butterfield's buffer solution (pH 7.2). Blending was done in a Stomacher 400 for 1 min. Prior to taking samples of flesh, the skin was washed with 70% ethanol and then removed aseptically. The underlying muscle was removed and after mincing, 25g were weighed into a stomacher bag containing 225 mL Butterfield's buffer solution. Blending was done in a Stomacher for 1 min. All cod and ocean perch fillets were handled in the same way as muscle samples. Butterfield's buffer was used for all dilutions.

When examining whole cod and cod fillets, bacterial counts were done by the pour plate technique on plate count agar (PCA-Difco) with 0.5% NaCl (w/v) added. The plates were incubated at 35°C for 2 days when counting mesophilic bacteria and for 22°C for 3 days when counting psychrotrophic bacteria. Counts of coliform bacteria were done by the MPN-technique (FDA, BAM, 1992). When examining ocean perch fillets, total viable counts and selective counts of H_2S-producing bacteria were done on iron agar (IA-Oxoid) as described by Gram et al. (1987). The plates were incubated at 22°C for 3 days. Bacteria forming black colonies on this agar produce H_2S from sodium thiosulphate and/or cysteine.

Composition of bacterial flora

On each occasion, 25 randomly selected colonies were picked off the PCA plates (cod fillets) and IA plates (ocean perch fillets). The following tests were used for determination of purified strains: Gram-staining (Hucker's modification) and morphology on young PCA cultures, KOH-

test (Gregersen, 1978), motility test on fresh cultures in Nutrient broth by the "hanging-drop" method, production of oxidase (Kovacs, 1956) and catalase (3% H_2O_2) and oxidation-fermentation test for glucose metabolism in MOF medium (Leifson, 1963) with 0.5% NaCl used instead of seawater salts. The tubes were incubated at 22°C and acid formation recorded after 7 and 14 days. When testing for TMAO-reduction, each strain was inoculated into 50 mL of 0.5% TMAO-broth and incubated at 22°C for 7 days. The TMAO-medium was prepared by adding 1.25 mL of 20% filter-sterilized trimethylamine N-oxide (Sigma) solution to 48.75 mL of sterile Nutrient broth. Reduction of TMAO was detected by measuring TMA (described below) and by sensory evaluation (smell). Identification of Gram-negative bacteria was based on the determinative scheme of Shewan et al. (1960) but updated according to *Bergey' Manual* Vol. 1 (Krieg and Holt, 1984). Gram-positive strains were classified according to *Bergey's Manual* Vol. 2 (Sneath et al., 1986).

Chemical analysis

TMA (as mgN/100g muscle) was measured according to AOAC (1990) except that KOH was used instead of K_2CO_3. TMAO (as mgN/100g muscle) was measured as described by Bystedt et al. (1959) where available TMA is first measured and then TMAO reduced to TMA by $TiCl_3$. Total volatile bases (TVB, as mgN/100g muscle) were measured according to Antonacopoulus (1968) with a Struer automatic distillation unit. TVB was only measured in ocean perch fillets.

Sensory evaluation

Sensory evaluation was by a trained panel of 7 to 10 people on samples of cooked fillets. Fillets were cooked in a steam oven (6 min at 98°C) and the smell and taste were judged on a rating scale from 9 (highest freshness) to 1 (lowest). The scale is based on the Torryscale as originally described by Shewan et al. (1953) with slight modifications. The fish were judged unfit for consumption when the mean value for sensory score was below 4.5.

Statistical analysis

Statistical analysis was done using SYSTAT 5.0 statistical package run on a PC computer. Analysis of variance was used and when appropriate Tukey test was used to find mean separation (Wilkinson, 1990). Linear regression was used on sensory data vs storage time in ice. Results from all bacterial counts were shown as geometric means of individual measurements but all other tests as arithmetic means.

RESULTS & DISCUSSION

Whole cod

TMA measurements in unfrozen and frozen-thawed cod during 3 wk storage in ice showed TMA formation was much slower in thawed whole cod (Fig. 1). On the 11th day and onwards there was a significant difference ($p < 0.05$) between means. The sensory panel gave a significant one point higher average score ($p < 0.05$) for the unfrozen cod at the beginning of storage than for thawed cod. Thereafter similar scores were given for both groups and after 15 to 16 days storage in ice, both unfrozen and thawed cod were unacceptable. At that time TMA in unfrozen cod was about 10 mgN/100g while TMA in thawed cod was around 1. Cod containing more than 10–15 mgN/100g is considered unsuitable for most uses (Connell, 1990). Growth curves of psychrotrophic bacteria for unfrozen and thawed cod were very similar. The difference between counts in skin (cm^2) and flesh (g) was in the range of 3 to 4 log numbers (Fig. 2).

Cod fillets in ≈2.25 kg cartons

Bacterial counts showed that the freezing (1 day in freezer) did not result in reduction of mesophilic and psychrotrophic bacteria or coliforms. However, with increasing storage time in freezer, the reduction in bacterial numbers became greater. Thus 50 wk storage resulted in 70–91% kill of bacterial flora (Table 1).

The percentage composition of psychrotrophic flora in fillets kept frozen for 23 wk (Table 2) showed the freezing process and 1 day storage in a freezer did not have a notable effect on composition. However, long term freezer storage (23 wk) resulted in an increased proportion of Gram-positive bacteria as expected (Shewan, 1961). The mean total count of six samples (Log no./g) was 5.1 (SD ± 0.1) in unfrozen fillets, 5.1 (SD ± 0.1) in fillets kept frozen 1 day and 4.8 (SD ± 0.2) in comparable fillets kept for 23 wk in a freezer. From the % proportion of TMAO-reducing bacterial strains the number of such bacteria was calculated and found to be Log 4.3 in unfrozen fillets, 4.0 in fillets kept frozen 1 day, and 3.7 in fillets kept 23 wk.

Table 1—Reduction in bacterial numbers in cod fillets in 2.25 kg cartons after different keeping time at −25°C

	% Reduction in bacterial numbers		
Storage time	Mesophiles	Psychrotrophs	Coliforms
1 day	0	0	0
18 wk	28	37	60
23 wk	53	43	80
50 wk	70	90	91

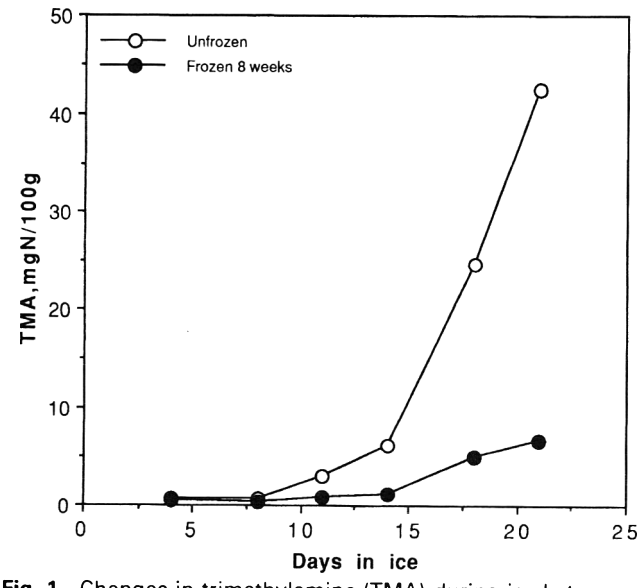

Fig. 1—Changes in trimethylamine (TMA) during iced storage of unfrozen and thawed whole cod (frozen 8 wks). Means of 4 samples.

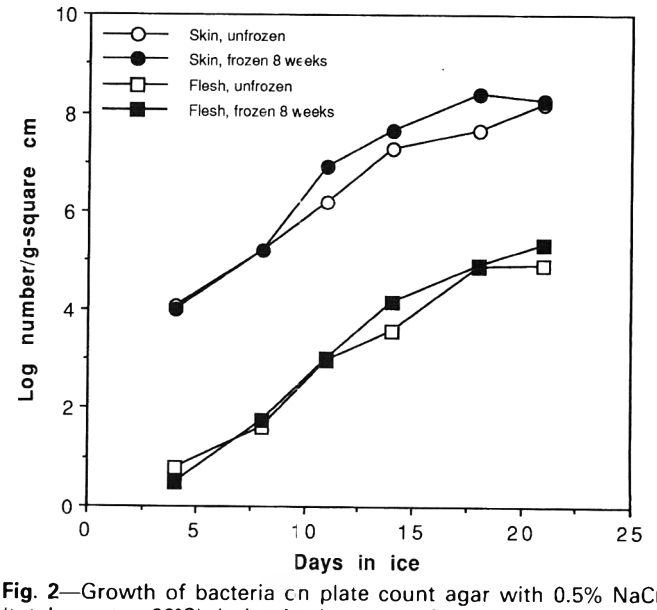

Fig. 2—Growth of bacteria on plate count agar with 0.5% NaCl (total count at 22°C) during iced storage of unfrozen and thawed whole cod (kept frozen 8 wks). Counts in both skin and flesh samples are shown. Means of 4 samples.

Table 2—Composition of the bacterial flora in cod fillets in ≈2.25 kg cartons (brackets show % proportion of TMAO reducing strains)

	% Composition of the psychrotrophic bacterial flora on PCA + 0.5% NaCl		
	Unfrozen	Frozen 1 day	Frozen 23 weeks
1. GRAM-positive	20	20	40
*Mic/Staph**	8(4)	8(4)	20(8)
Coryneforms	12(4)	12	20
2. GRAM-negative	72	80	52
*Mor/Aci**	52(8)	36(4)	32
*Flav/Cyt**	20	40	20
*Ps/Alt/Alc**	0	4	0
3. Unidentified	8	0	8

* Mic/Staph = *Micrococcus/Staphylococcus*, Mor/Acin = *Moraxella/Acinetobacter*, Flav/Cyt = *Flavobacterium/Cytophaga*, Ps/Alt/Alc = *Pseudomonas/Alteromonas/Alcaligenes*

Results from TMAO measurements in fillets kept up to 50 wk in a freezer indicated that TMAO was not broken down during freezer storage. Thus, TMAO (as mg N/100g) was 82.4 (SD ± 7.1) in unfrozen fillets, 82.1 (SD ± 8.5) in fillets kept frozen 1 day, 85.1 (SD ± 7.6) in fillets kept frozen 23 wk and 81.2 (SD ± 0.8) in fillets kept frozen 50 wk. Differences in means were not significant ($p > 0.05$).

Cod fillets

No significant difference was found between the initial number of psychrotrophic bacteria in unfrozen cod fillets, those kept frozen 1 day or 5 wk. However numbers of bacteria were lower ($p < 0.05$) when fillets had been kept in frozen storage for 14, 27 and 52 wk (Fig. 3). Most of the time in iced storage, numbers of bacteria were lower in those fillets that had been kept longest in a frozen state (Fig. 3). Differences in bacterial counts after 7, 10 and 14 days in ice were significant between fillets that had been kept for < 14 wk in a frozen state and those kept > 14 wk. After 17 days iced storage the difference was not significant.

The proportion of Gram-positive bacteria did not increase with increasing frozen storage as had been found for cod fillets in cartons (Table 3). However, during storage in ice, Gram-negative bacteria became predominant with the highest proportion of the genera *Pseudomonas/Alteromonas/Alcaligenes.* No marked difference in bacterial flora of unfrozen fillets occurred during storage in ice and frozen-thawed fillets.

From the % proportion of TMAO-reducing bacterial strains the number of such bacteria after 1 day storage in ice was calculated to be Log 4.3 in unfrozen fillets, 4.2 in those kept frozen 1 day and 3.4 in fillets kept frozen 27 wk. This corresponded to the TMAO-reducing bacteria in thawed fillets (kept frozen 27 wk) being 12% of the number of such bacteria in unfrozen fillets. During prolonged iced storage the number of TMAO-reducing bacteria was lower in thawed fillets than in unfrozen ones. Thus, after 10 days storage in ice, the number of TMAO-reducing bacteria was log 5.3 in unfrozen fillets but 4.7 in those kept frozen 27 wk. Calculated as percentages this corresponded to the TMAO-reducing bacteria in thawed fillets (kept frozen 27 wk) being 25% of the number in unfrozen fillets.

TMA measurements showed that average TMA had not exceeded 2 mgN/100g after 13 days storage in ice, even in unfrozen fillets. At the end of storage or after 21 days in ice the TMA level in unfrozen fillets was 16.3 (Fig. 4). The longer the

Table 3—Composition of the bacterial flora in cod fillets (numbers in brackets show % proportion of TMAO reducing strains)

	% Composition of the psychrotrophic bacterial flora on PCA + 0.5% NaCl								
	Unfrozen			Frozen 1 day			Frozen 27 wk		
	1 day in ice	10 days in ice	21 days in ice	1 day in ice	10 days in ice	21 days in ice	1 day in ice	10 days in ice	21 days in ice
1. GRAM-positive	48	16	12	24	24	12	44	12	0
*Mic/Stap**	8(4)	8	8	8(8)	12(4)	8	24(4)	0	0
Coryneforms	40(8)	8	4	16(4)	12	4	20(4)	12(4)	0
2. GRAM-negative	48	80	80	76	76	88	44	88	100
*Mor/Acin**	12	72(4)	28	20	40	8	20	56(4)	24
*Flav/Cyt**	28	0	8	48	0	4	20	4	0
*Ps/Alt/Alc**	8(4)	8	44(16)	8	36	76(16)	4	28	76
3. Unidentified	4	4	8	0	0	0	8	8	0

* Mic/Staph = *Micrococcus/Staphylococcus*, Mor/Acin = *Moraxella/Acinetobacter*, Flav/Cyt = *Flavobacterium/Cytophaga*, Ps/Alt/Alc = *Pseudomonas/Alteromonas/Alcaligenes*

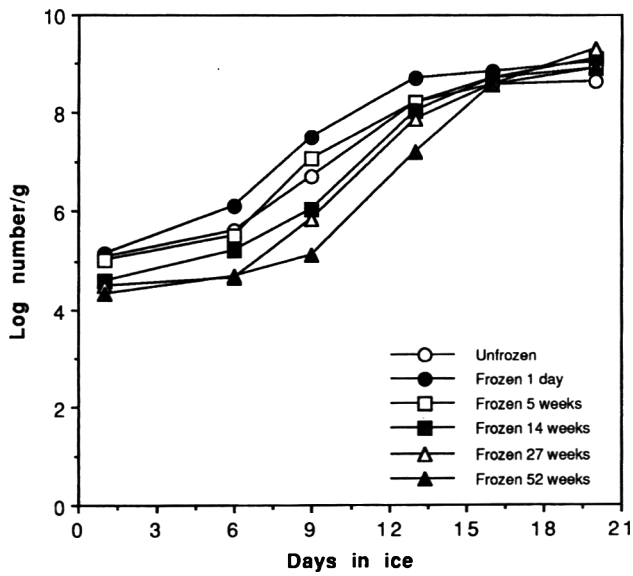

Fig. 3—Growth of bacteria on plate count agar with 0.5% NaCl (total count at 22°C) during iced storage of unfrozen and thawed cod fillets (kept frozen 1 day and 5, 14, 27 and 52 wks). Means of 3 samples.

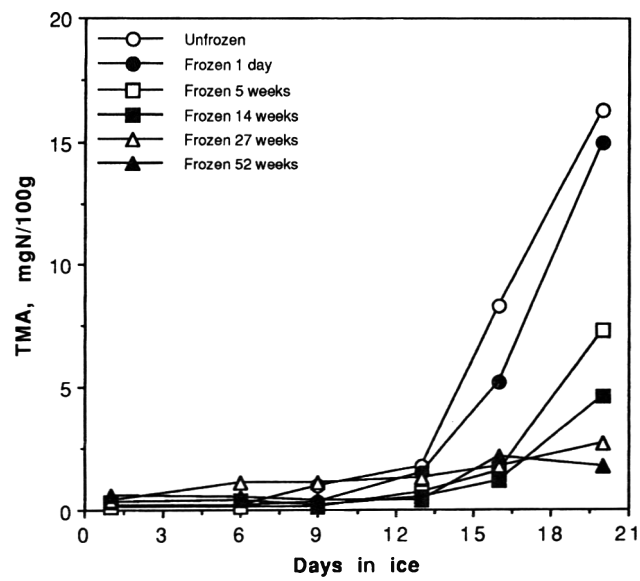

Fig. 4—Changes in trimethylamine (TMA) during iced storage of unfrozen and thawed cod fillets (kept frozen 1 day and 5, 14, and 27 and 52 wks). Means of 3 samples.

fillets had been kept frozen the slower the TMA formation during iced storage. Much more TMA was formed in the flesh of whole cod than in fillets kept on ice, reaching values > 40 after 21 days iced storage (Fig. 1). The reduction of TMAO to TMA was expected to proceed at a faster rate at conditions of low oxygen tension (Huss, 1972). The spoilage bacteria use TMAO as an electron acceptor instead of oxygen (anaerobic respiration) when oxygen is in low concentration. This leads to more TMA production in whole cod than in iced fillets where oxygen concentration would be expected to be much higher. We also expected that the skin would prevent leakage of TMAO from the flesh with the melt water from the ice.

Results from TMAO measurements in fillets kept up to 52 wk in a freezer showed that it was not broken down during freezer storage. Thus, TMAO (as mg N/100g) was 90.1 (SD ± 6.6) in unfrozen fillets, 83.3 (SD ± 9.4) in fillets kept frozen 5 wk, 85.1 (SD ± 12.3) in fillets kept frozen 14 wk, 78.2 (SD ± 3.0) in those kept frozen 27 wk and 94.9 (SD ± 4.3) in fillets kept frozen 52 wk.

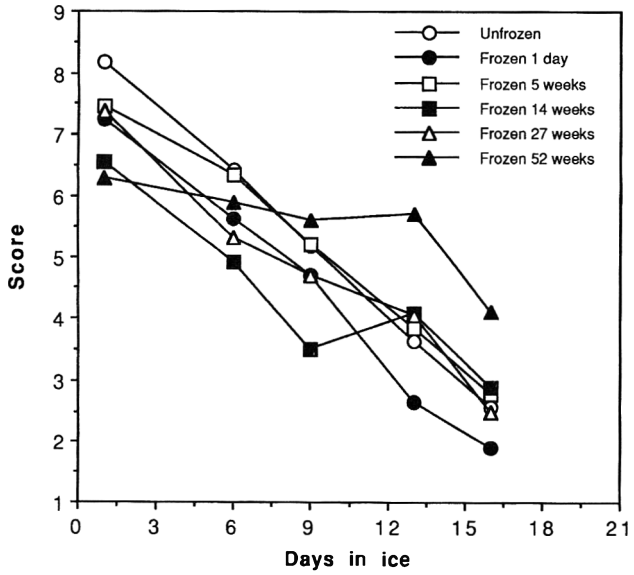

Fig. 5—Sensory evaluation during iced storage of unfrozen and thawed cod fillets (kept frozen 1 day and 5, 14, 27 and 52 weeks). Means of 3 samples.

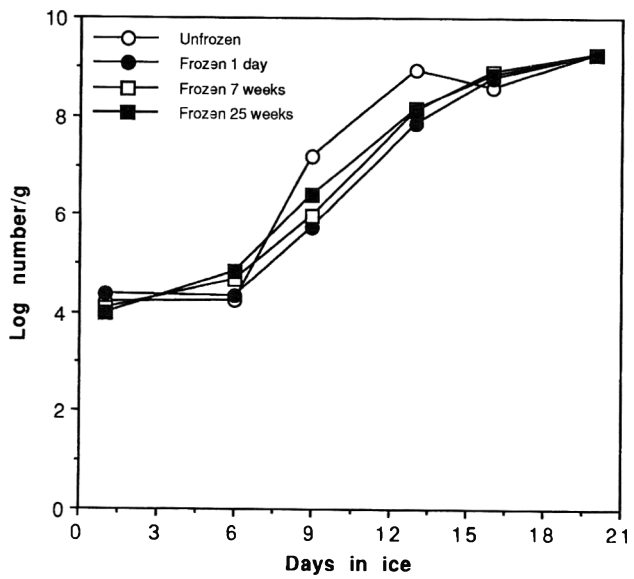

Fig. 6—Growth of bacteria on iron agar (total count at 22°C) during iced storage of unfrozen and thawed ocean perch fillets (kept frozen 1 day, 7 weeks and 25 weeks). Means of 3 samples.

Table 4—Linear regression of sensory scores for cod fillets vs days in ice (Y = a + b X (days in ice))

Storage time	Intercept (a)	Slope (b)	Coefficient of determination (r^2)
Unfrozen	8.33	−0.36	0.995
Frozen 1 day	7.49	−0.35	0.983
Frozen 5 wk	7.76	−0.30	0.983
Frozen 14 wk	6.63	−0.21	0.875
Frozen 27 wk	7.27	−0.28	0.971

Sensory evaluation showed that at the beginning of iced storage the unfrozen fillets had highest ($p < 0.05$) freshness scores (Fig. 5). Fillets kept 52 wk in frozen state were lower. Assuming linear regression (except for fillets kept 52 wk) between scores and storage time in ice the equations for the lines were calculated (Table 4) and maximum storage time in ice found using 4.5 as a borderline. In all groups except fillets kept for a year, the keeping time in ice was around 10–12 days. The fillets kept frozen for 52 wk had average scores between 5 and 6 most of the storage time. On the 14th day in ice those fillets had higher ($p < 0.05$) scores than those in all other experimental groups. By the time the panel judged the cod fillets unacceptable the TMA had not reached 2 mgN/100g. This applied both to unfrozen and thawed fillets.

Ocean perch fillets

Total counts on IA showed there was no significant difference in initial numbers of psychrotrophic bacteria in unfrozen and thawed fillets. Over 20 days storage the increase in numbers of bacteria were similar in all groups except that for 9 and 13 days in ice higher numbers were obtained on the unfrozen fillets (Fig. 6). Numbers of H_2S-producing bacteria on IA were low during storage. On average they were about 1% of the total count. No significant difference occurred in initial numbers of H_2S-producing bacteria in unfrozen and thawed fillets. However, the number of these bacteria was higher in unfrozen fillets on days 6, 9 and 13 of iced storage (Fig. 7).

A slight increase in proportion of Gram-positive bacteria was noticed during frozen storage (Table 5). In the beginning of iced storage, bacteria belonging to *Vibrio/Aeromonas* were most predominant in unfrozen fillets while in thawed fillets *Micrococcus/Staphylococcus* were most predominant. However, during iced storage, Gram-negative bacteria became predominant with high-

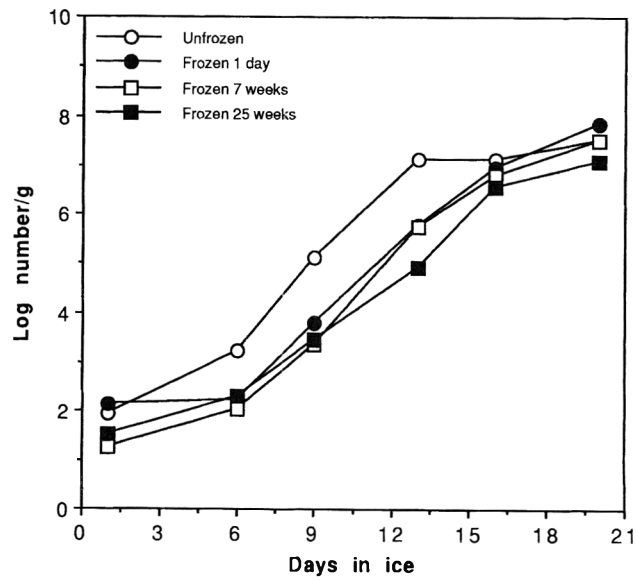

Fig. 7—Growth of H_2S-producing bacteria on iron agar (count at 22°C) during iced storage of unfrozen and thawed ocean perch fillets (kept frozen 1 day, 7 weeks and 25 weeks). Means of 3 samples.

Table 5—Composition of the bacterial flora in ocean perch fillets (numbers in brackets show % proportion of TMAO reducing strains)

	% Composition of the psychrotrophic bacterial flora on iron agar					
	Unfrozen		Frozen 1 day		Frozen 25 wk	
	1 day in ice	16 days in ice	1 day in ice	16 days in ice	1 day in ice	16 days in ice
1. GRAM-positive	44	0	60	16	52	8
*Mic/Staph**	4(4)	0	28(20)	0	40(20)	0
Coryneforms	16	0	12	12	8	8
Lactobacillus	12	0	4	4	4	0
*Ped/Strep/Leu**	12	0	16	0	0	0
2. GRAM-negative	52	100	36	80	40	92
*Mor/Acin**	16	0	4	0	20	20
*Flav/Cyt**	4	0	12	0	12	0
*Ps/Alt/Alc**	0	100(8)	0	80(12)	0	72(4)
Ente-obacteriaceae	4	0	20	0	4(4)	0
Vibrio/Aeromonas	28(4)	0	0	0	4	0
3. Unidentified	4	0	4	4	8	0

* Mic/Staph = *Micrococcus/Staphylococcus*, Ped/Strep/Leu = *Pediococcus/Streptococcus/Leuconostoc*, Mor/Acin = *Moraxella/Acinetobacter*, Flav/Cyt = *Flavobacterium/Cytophaga*, Ps/Alt/Alc = *Pseudomonas/Alteromonas/Alcaligenes*.

est proportion of the genera *Pseudomonas/Alcaligenes/Alteromonas*. From the % proportion of TMAO-reducing bacterial strains the number of such bacteria after 1 day storage in ice was calculated to be Log 3.2 in unfrozen, 3.7 in fillets kept frozen 1 day and 3.4 in those kept frozen 25 wk. The number of these bacteria was not lower in thawed fillets than in unfrozen fillets during prolonged iced storage.

Thirteen of the isolated colonies from IA were black (H_2S-producing). These were identified as *Pseudomonas/Alteromonas/Alcaligenes* (8), *Vibrio/Aeromonas* (4) and *Enterobacteriaceae* (1). Only 6 of these 13 strains or 46% were able to reduce TMAO. These were *Pseudomonas/Alteromonas/Alcaligenes*. Members belonging to these genera were 62% of the total number of black colonies. This shows that not all strains which form black colonies on IA reduce TMAO and that other species than those belonging to *Pseudomonas/Alteromonas/Alcaligenes* can form black colonies. Furthermore 75% of bacteria identified as *Pseudomonas/Alteromonas/Alcaligenes* reduced TMAO. Some strains which belong to these genera therefore do not reduce TMAO. We could assume that at least some of the strains referred to as *Pseudomonas/Alteromonas/Alcaligenes* belong to the species *Alteromonas putrefaciens*, now commonly named *Shewanella putrefaciens* (MacDonell and Colwell, 1985). This species has frequently been reported as a main spoilage organism in fish.

After 16 days iced storage TMA had not yet reached 1 mgN/100g in any experimental groups. It was not until after 20 days in ice that difference was noticed in TMA values. Highest average values were found in unfrozen fillets or 6.8 (SD ± 1.4). Comparable values were 4.1 (SD ± 1.1) in fillets kept frozen 1 day, 3.5 (SD ± 0.3) in fillets kept frozen for 7 wk and 1.9 (SD ± 0.3) in fillets kept frozen 25 wk (Fig. 8). This difference was significant ($p < 0.05$) between unfrozen and thawed fillets. Results were in agreement with earlier results when examining TMA formation in frozen-thawed cod.

Analysis of TVB (mgN/100g, Fig. 9) showed in all experimental groups the TVB during the first 16 days storage had an average reduction of 28%. The explanation could be that some of the volatile bases leaked away with the melt water from the ice. Not until the 20th day of iced storage was an increase in TVB observed. The highest increase was in unfrozen fillets and there was a significant difference ($p < 0.05$) in TVB between unfrozen fillets (33 ± 3.2) and those kept frozen for 25 wk (20.8 ± 4.5).

TMAO (Fig. 10) showed initial values from 87.9 to 96.5 mgN/100g and were not significantly different ($p > 0.05$) between experimental groups. This indicated that TMAO had not broken down in the fillets during frozen storage. In all experimental groups TMAO decreased during storage in ice by up to 81%. This loss could not be related to bacterial reduction of TMAO as TMA was measured under 1 mgN/100g most of the storage time. The most likely explanation is that TMAO had leaked away with the melt water from the ice. The TMAO loss was not significantly lower in frozen-thawed fillets than in un-

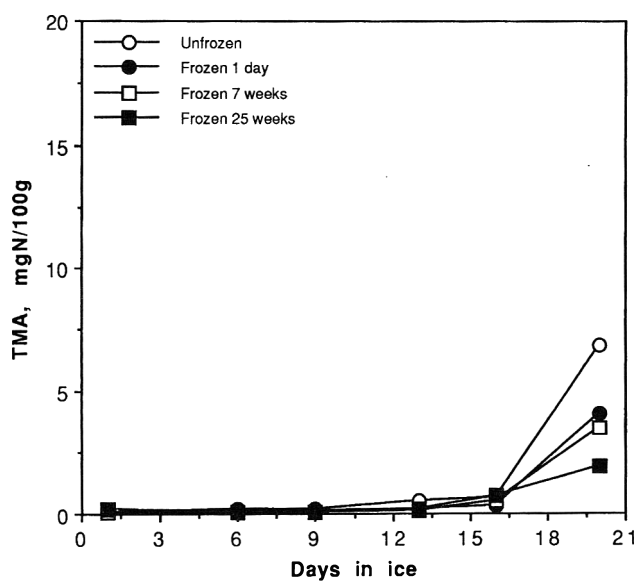

Fig. 8—Changes in trimethylamine (TMA) during iced storage of unfrozen and thawed ocean perch fillets (kept frozen 1 day, 7 weeks and 25 weeks). Means of 3 samples.

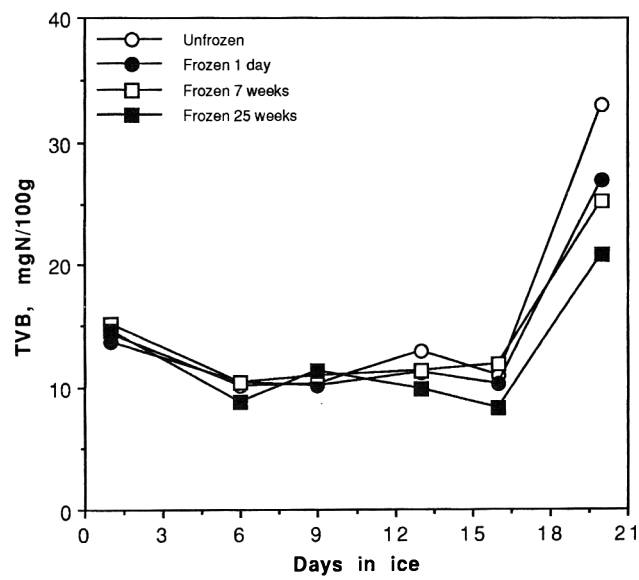

Fig. 9—Changes in total volatile bases (TVB) during iced storage of unfrozen and thawed ocean perch fillets (kept frozen 1 day, 7 weeks and 25 weeks). Means of 3 samples.

Table 6—Linear regression of sensory scores for ocean perch fillets versus days in ice (Y = a + b X (days in ice))

Storage time	Intercept (a)	Slope (b)	Coefficient of determination (r^2)
Unfrozen	8.76	−0.43	0.888
Frozen 1 day	7.71	−0.33	0.893
Frozen 7 wk	7.49	−0.33	0.917
Frozen 25 wk	6.56	−0.20	0.580

frozen fillets (as might have been expected) due to possible cell damage during freezing, freezer-storage and thawing.

Sensory evaluation showed that at the beginning of iced storage unfrozen fillets had significantly highest scores (Fig. 11). The unfrozen samples obtained higher scores throughout most of the storage period. Assuming linear regression (except for fillets kept 52 wk) between scores and storage time in ice equations were calculated (Table 6) and maximum storage time in ice was found using 4.5 as a borderline. On the 9th to 10th day all experimental groups had reached about the limit of edibility. By the time the panel judged the ocean perch fillets unacceptable the TMA was < 1 mgN/100g. This applied both to unfrozen and thawed fillets.

CONCLUSIONS

FREEZING AND SHORT-TERM FREEZER storage (≤5 wk) has little effect on bacterial counts. Thus, bacterial counts obtained from frozen samples kept for a short time in freezer reflect bacterial numbers just prior to freezing. However, during long-term freezer storage (≥14 wk) there was a reduction in total counts and counts of TMAO-reducing bacteria in cod fillets but not ocean perch fillets. The proportion of Gram-positive bacteria did not increase distinctly with increasing frozen storage as expected except in cod fillets kept in 2.25 kg cartons. However, during storage in ice, Gram-negative bacteria became predominant in both cod and ocean perch fillets. The numbers of H_2S-producing bacteria in ocean perch fillets were low throughout storage. Sensory evaluation showed that thawed fillets never had as high scores at the beginning of iced storage as unfrozen fillets. However, similar scores were found for both unfrozen and thawed fillets after 10–12 days in ice when they were unacceptable. The longer the fillets had been kept frozen, the slower the TMA and TVB formation in the fillets during iced storage. Use of TMA as a spoilage indicator is of no value when evaluating spoilage state of unfrozen and frozen-thawed cod and ocean perch fillets kept in ice.

REFERENCES

Antonacopoulus, N. 1968. In *Handbuch der Lebensmittelchemie*, Vol. III/2. Springer Verlag, Berlin.

AOAC. 1990. *Official Methods of Analysis*, 15th ed. Association Official Analytical Chemists, Washington, DC.

Bystedt, J., Swenne, L., and Aas, H.W. 1959. Determination of trimethylamine oxide in fish muscle. J. Sci. Food Agric. 10: 301–304.

Castell, C.H., Smith, B., and Dyer, W.J. 1974. Simultaneous measurements of trimethylamine and dimethylamine in fish, and their use for estimating quality of frozen-stored gadoid fillets. J. Fish. Res. Board Can. 31 (4): 383–389.

Connell, J.J. 1990. *Control of Fish Quality*, 3rd ed. Fishing News (Book) Ltd., London.

Food & Drug Administration. 1992. *Bacteriological Analytical Manual*, 7th ed. AOAC International, Arlington.

Gram, L., Trolle, G., and Huss, H.H. 1987. Detection of specific spoilage bacteria from fish stored at low (0°C) and high (20°C) temperatures. Int. J. Food Microbiol. 4: 65–72.

Gregersen, T. 1978. Rapid method for destinction of Gram-negative from Gram-positive bacteria. Eur. J. Appl. Microbiol. 5: 123–127.

Hebard, C.E., Flick, G.J., and Martin, R.E. 1982. Occurrence and significance of trimethylamine oxide and its derivatives in fish and shellfish. Ch. 12, in *Chemistry & Biochemistry of Marine Food Products*, R.E. Martin, G.J. Flick, C.E. Hebard, and D.R. Ward (Ed.), p. 149–304. AVI Publishing Company, Westport, CT.

Huss, H.H. 1972. Storage life of prepacked wet fish at 0°C. 1. Plaice and haddock. J. Food Technol. 7: 13–19.

Kovacs, N. 1956. Identification of *Pseudomonas pyocyanea* by the oxidase reaction. Nature 178: 703.

Krieg, N.R. and Holt, J.G. (Ed.). 1984. *Bergey's Manual of Systematic Bacteriology*, Vol. 1. Baltimore: Williams & Wilkins.

Leifson, E. 1963. Determination of carbohydrate metabolism of marine bacteria. J. Bacteriol. 85: 1183–1184.

Luijpen, A.F.M.G. 1958. Objective spoilage tests for fish stored under conditions other than normal chilling in ice. J. Sci. Food Agric. 9: 410–417.

MacDonell, M.T. and Colwell, R.R. 1985. Phylogeny of the Vibrionaceae, and recommendation for two new genera, *Listonella* and *Shewanella*. System. Appl. Microbiol. 6: 171–182.

Shewan, J.M. 1961. The microbiology of seawater fish. In *Fish as Food*, Vol. 1, G. Borgstrom (Ed.). Academic Press, London.

Shewan, J.M., MacIntosh, R.G., Tucker, C.G., and Ehrenberg, A.S.C. 1953. The development of a numerical scoring system for the sensory assessment of the spoilage of wet white fish stored in ice. J. Sci. Food Agric. 4: 283–298.

Shewan, J.M., Hobbs, G., and Hodgkiss, W. 1960. A determinative scheme for the identification of certain genera of Gram-negative bacteria, with special reference to the Pseudomonadaceae. J. Appl. Bact. 23: 379–390.

Sneath, P.H.A., Mair, N.S., Sharpe, M.E., and Holt, J.G. (Ed.). 1986. *Bergey's Manual of Systematic Bacteriology*, Vol. 2. Williams & Wilkins, Baltimore, MD.

Wilkinson, L. 1990. *SYSTAT: The System for Statistics*, Systat Inc., Evanston, IL.

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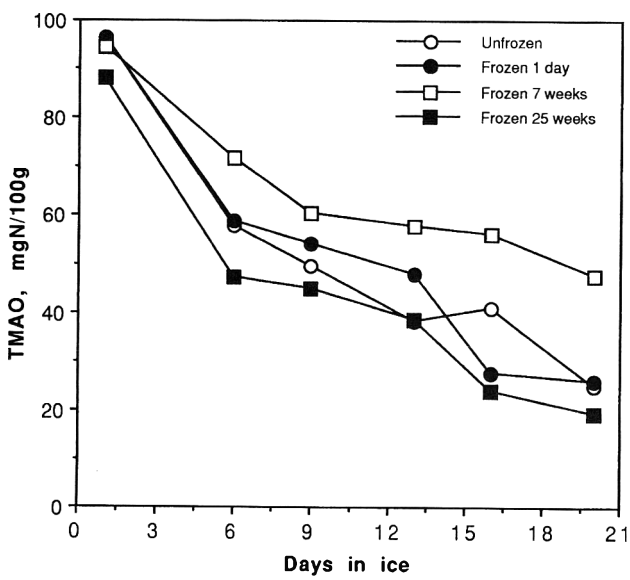

Fig. 10—Changes in trimethylamine oxide (TMAO) during iced storage of unfrozen and thawed ocean perch fillets (kept frozen 1 day, 7 weeks and 25 weeks). Means of 3 samples.

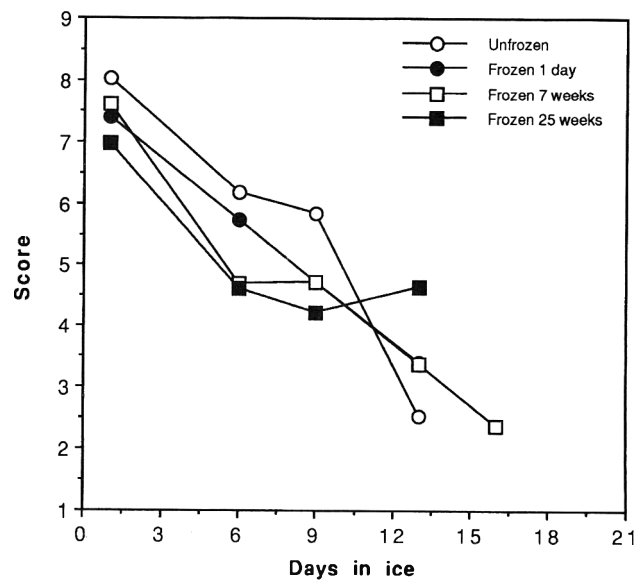

Fig. 11—Sensory evaluation during iced storage of unfrozen and thawed ocean perch fillets (kept frozen 1 day, 7 weeks and 25 weeks). Means of 3 samples.

Identification of Red Snapper (*Lutjanus campechanus*) using Electrophoretic Techniques

TUNG-SHI HUANG, MARTY R. MARSHALL and CHENG-I WEI

ABSTRACT

Isoelectric focusing (IEF), sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and two-dimensional (2-D) gel electrophoresis were employed to produce protein profiles for species identification of red snapper and 11 other fish species. Comparing the distinctive patterns of water-soluble sarcoplasmic proteins for each species on IEF and SDS gels, red snapper could be identified. IEF gels of ampholyte mixture of 20% pH 3–10 and 80% pH 4–6.5 resolved better than gels with ampholyte ranging from pH 3–10, 4–6.5, 6–8, or 5–6 for species identification. The 10 and 12.5% SDS-PAGE gels produced more distinctive protein profiles for identification than 7.5 and 15% gels. Thus, these techniques could be applied to identify fish species.

Key Words: red snapper, protein profiles, electrophoresis

INTRODUCTION

RED SNAPPER (*Lutjanus campechanus*) is one of the most important commercial fish in the snapper-grouper fishery in the Northern Gulf of Mexico (FAO Species Catalogue, 1985). It is a popular eating fish with relatively high market value. Because of their solitary habits and territorial behavior, red snapper are not caught in huge quantities. The yield of red snapper around Florida waters from about 1984 to 1994 has become nearly steady and is estimated to be 1,360–1,814 metric tons annually.

The high value of red snapper and the lack of predicted increases in this resource lead to illegal sale of other red snapper-like fish and/or the substitution of less valuable species in seafood markets. Illegal substitutions have occurred for red snapper and other valuable snappers and groupers and to a lesser extent, for mackerel and swordfish. The FDA (1980) issued a 'Compliance Policy Guide' (7108.21) to resolve concerns for selling Pacific coast rockfish (family *Scorpaenidae*) as red snapper. The development of analytical methods to identify fish species is, therefore, important in preventing willful or unintentional substitution of lower valued fish species for high valued fish in the marketplace.

Electrophoretic methods such as starch gel-zone electrophoresis, acrylamide disc electrophoresis, thin layer polyacrylamide gel isoelectric focusing and cellulose acetate strip have been accepted as official methods by the Association of Official Analytical Chemists (AOAC, 1990) to differentiate seafood species or seafood products. Lundstrom (1980) and An et al. (1988) utilized various electrophoretic support matrixes to successfully separate fish muscle proteins for identification. Sodium dodecyl sulfate (SDS) has been incorporated into the gel to improve resolution of electrophoretic protein patterns (Lundstrom 1979, 1980; Melvin, 1987; An et al., 1988).

Isoelectric focusing (IEF) has been extensively used for identification of seafood species because it provides reliable and reproducible resolved protein patterns for differentiating closely related species (Lundstrom, 1983a, 1983b; An et al., 1989). Urea has also been incorporated to enhance protein separation and resolution (Keung et al., 1985; An et al., 1989). The Florida Department of Agriculture and Consumer Services used thin layer IEF to identify retail snapper fillets (Hsieh et al., 1989).

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Our objective was to investigate the feasibility of using SDS-polyacrylamide gel electrophoresis (SDS-PAGE), IEF, and two-dimensional (2-D) gel electrophoresis to prepare protein profiles for red snapper identification compared with other closely related fish species that may be substituted illegally for red snapper.

MATERIALS & METHODS

Fish samples

Authentic samples of red snapper (*Lutjanus campechanus*, RS), vermilion snapper (*Rhomboplites aurorubens*, VS), gray snapper (*Lutjanus griseus*, GS), hogfish (*Lachnolaimus maximus*, HF), lane snapper (*Lutjanus synagris*, LS), mutton snapper (*Lutjanus analis*, MS), and yellowtail snapper (*Ocyurus chrysurus*, YS) were obtained from the Food Laboratory, Florida Department of Agriculture and Consumer Service, Tallahassee, FL. Red snapper obtained from the Texas and Florida coasts off the Gulf of Mexico were provided by Dr. W. S. Otwell, University of Florida. Onespot snapper (*Lutjanus monostigma*, OS), blackspot snapper (*Lutjanus fulviflamma*, BS), and Madras snapper (*Lutjanus lutjanus*, LL) were obtained from Taiwan and shipped in dry ice to the laboratory at the Food Science and Human Nutrition Dept., Univ. of Florida. VS, GS, HF, pink porgy (*Pagrus pagrus*, PG), and white grunt (*Haemulon plumieri*, WG) were purchased from a local seafood store. Each species was represented by at least two fish. Fish filets were prepared after they were scaled, gutted, and headed. The filets (white muscle only) were then cut into small pieces of about 20g, put in Whirlpak® bags, and stored at −33°C until needed.

Protein extraction and sample preparation

The outer layers of defrosted fish samples were removed. Only the center part of each sample, about 6g, was homogenized with 3 volumes of water (w/v) at 24°C for 1 min using a Polytron (setting 6.2, Brinkmann Instruments, Westbury, NY). The water contained 0.1 mM phenylmethylsulfonyl fluoride (PMSF), 10 mM EDTA and 0.01% sodium azide to inhibit proteases and microbial growth. After homogenized samples were centrifuged at 26,900 × *g* for 20 min at 5°C, the supernatants were collected and protein concentrations determined by Lowry method (Lowry et al., 1951). Following protein adjustment to 5 mg/mL with water, the supernatants were added with sucrose granules to contain 6% sucrose. The sample preparations were then placed into small vials in 100 µL aliquots, and stored at −70°C.

Isoelectric focusing electrophoresis (IEF)

A gel mixture containing 4% (w/v) acrylamide (containing 5.3% cross-linker N'N'-bis-methylene-acrylamide [Bis]), 2% (w/v) Triton X-100 and 9.2 M urea was mixed for 5 min at 37°C. Following addition of ampholyte (Pharmacia, Piscataway, NJ) mixture containing 20%, pH 3–10 and 80%, pH 4–6.5 (Wei et al., 1990) to a final concentration of 2% (v/v), the gel mixture was degassed for 3 min. After adding fresh ammonium persulfate (0.02%, v/v) and N,N,N',N'-tetramethylethylenediamine (TEMED, 0.14%, v/v), the mixture was poured into 16 × 20 cm slab gel plates (0.75 mm thick) assembled with a comb. The gel was then allowed to polymerize for 2 hr. Following removal of the comb from the gel, lysis buffer containing 9.5 M urea, 2% Triton X-100 and 2% (v/v) ampholyte (pH 4–6.5) was overlaid on the gel for 1 hr. The gel was prefocused at 200 V for 15 min, then increased to 300 V for 30 min and 400 V for a final 30 min using 0.01 M phosphoric acid as the anode solution and 0.02 M sodium hydroxide as the cathode solution. After prefocusing, protein samples (100 µg/well) were applied to the gel and overlaid with an aqueous solution containing 2% Triton X-100 and 2% ampholyte (pH 4–6.5). The gel plate was then reassembled in the electrophoresis unit; and after fresh cathode solution was added to the chamber, proteins were focused at 24°C for 17 hr at 400 V with circulating tap water. After the IEF run, gels were fixed in a fixative (4% sulfosalicylic acid and 12.5% trichloroacetic acid) for 6 hr, stained with

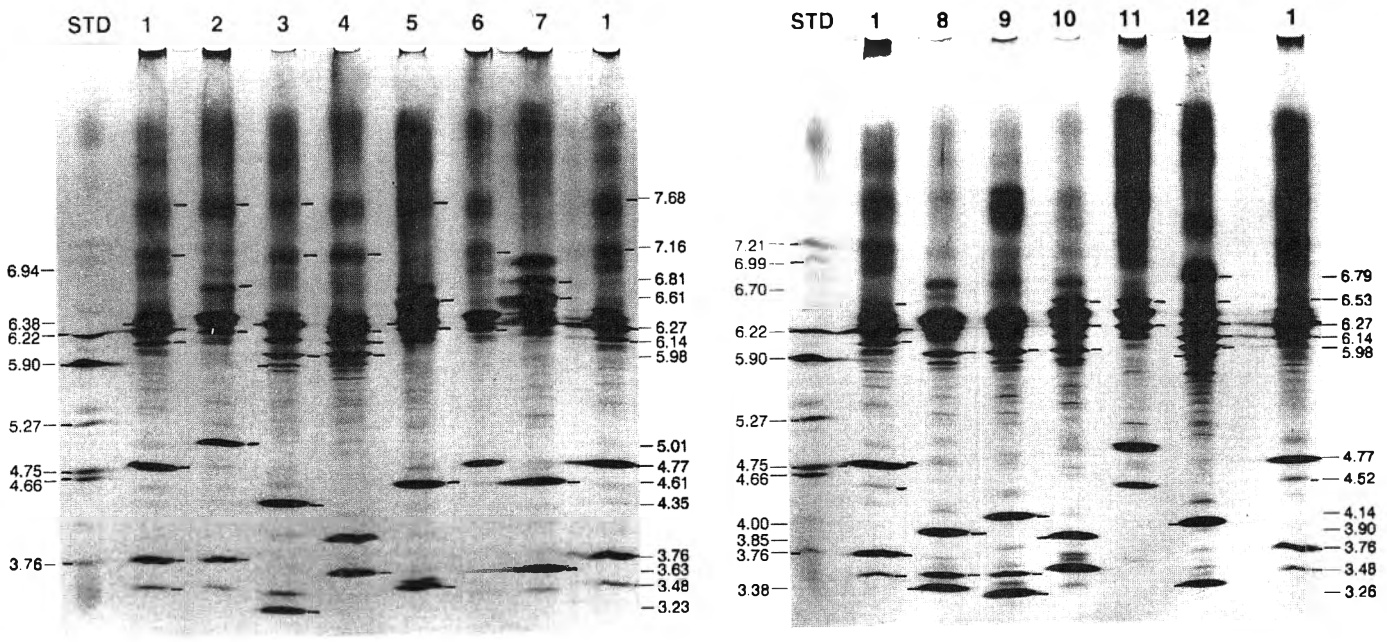

Fig. 1—IEF protein patterns of water-soluble muscle sarcoplasmic extracts from (1) RS, (2) VS, (3) GS, (4) HF, (5) LS, (6) MS, (7) YS, (8) OS, (9) BS, (10) LL, (11) PG, and (12) WG on pH 3---10 electrophoresis gels. The numerical designations indicate apparent pI values of the protein bands.

0.04% Coomassie blue R-250 for 1 hr, destained in a solution containing 0.5% copper sulfate, 12% 2-isopropanol, and 7% acetic acid, and then stored in a 7% acetic acid - 5% methanol solution. Electrophoretic patterns were recorded by developing the positive image using a Kodak Electrophoresis Duplicating Paper (Eastman Kodak Co., Rochester, NY).

IEF gels of differing pH ranges were also prepared to evaluate fish protein patterns using ampholytes giving pH of 3–10, 4–6.5, 6–8, and 5–6). Running conditions and chemicals used were as described.

Apparent pI of protein bands

The apparent pI values of fish proteins were determined indirectly by comparing their R_f values on the gel with those of protein standards (Broad pI kit, pH 3–10, Pharmacia) containing trypsinogen, pI 9.30; lentil lectin-basic band, pI 8.65; -middle band, pI 8.45; -acidic band, pI 8.15; horse myoglobin-basic band, pI 7.35, -acidic band, pI 6.85; human carbonic anhydrase B, pI 6.55; bovine carbonic anhydrase, pI 5.85; β-lactoglobulin A, pI 5.20; soybean trypsin inhibitor, pI 4.55; and amyloglucosidase, pI 3.50. From apparent pI and R_f values of the standards and the R_f values of fish proteins on the gel, the apparent pI of fish proteins was determined (Låås et al., 1980; An et al., 1989).

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE was performed according to the modified procedures of Laemmli (1970) and O'Farrell (1975) using a Protean unit (Bio-Rad, Hercules, CA) (An et al., 1988). SDS-PAGE gels were stained with 0.125% (w/v) Coomassie Brilliant Blue R-250 in 40% ethanol and 7% acetic acid, and then destained in 7% acetic acid containing 5% methanol. Electrophoretic patterns were recorded as described. Molecular weights were determined by comparing relative mobilities of protein bands to standard proteins (Weber et al., 1972). Low molecular weight (MW) proteins (Pharmacia) containing phosphorylase b (MW, 94 kD), albumin (67 kD), ovalbumin (43 kD), carbonic anhydrase (30 kD), trypsin inhibitor (20.1 kD), and α-lactalbumin (14.4 kD) were used as standards.

Two-dimensional (2-D) electrophoresis

Isoelectric focusing gels were made in glass tubes (140 × 1.5 mm inside diameter, Bio-Rad) sealed at the bottom with Parafilm. The gel solution which contained 20% pH 3–10 and 80% pH 4–6.5 ampholyte, and the remaining ingredients described in the IEF gel electrophoresis section were loaded into gel tubes using a syringe with a long narrow gauge hypodermic needle to about 5 mm from the top. Gels were overlaid with 8 M urea solution, and 1 hr later, this overlay solution was removed and replaced with lysis buffer. The gels were allowed to set for 1 more hr. After removing the Parafilm, the tubes were placed in a tube gel electrophoresis chamber (Bio-Rad). The lysis buffer was removed from the surface of the gel and fresh lysis buffer was added. The lower reservoir was filled with 0.01M H_3PO_4, and the upper reservoir filled with 0.02 M NaOH, both being extensively degassed. The gels were then prefocused at 200 volts for 15 min, 300 volts for 30 min, and then 400 volts for 30 min. Following prefocusing, the power was turned off and the upper reservoir emptied. The lysis buffer and NaOH solution were removed from the surface of the gels, and samples were loaded with a micropipette. The gels were then run at 24°C for 17 hr at 400 volts with circulating water. Gels were slowly forced out by pressure from the tubes with a 20-mL syringe connected to the electrophoresis tube via a short piece of Tygon™ tubing.

The SDS-PAGE slab gels consisting of a 15% running (containing 2.7% Bis) and a 3.1% stacking gel (containing 2.7% Bis) were used in the second dimension. A 2-D gel comb was inserted 8 mm below the notch to form a flat surface on the top of the stacking gel. After the gel solution was polymerized, the comb was removed from the gel and all unpolymerized gel solution removed from the surface. The first dimensional IEF tube gel was then placed on a piece of Parafilm and straightened. One mL of 1% melted agarose solution was put in the notch to keep the IEF gel in place and avoid mixing as the protein zones migrated out of the cylinder into the slab gel. The IEF gel was then placed parallel to and near one edge of the Parafilm and transferred onto the top of the SDS slab gel.

To avoid protein losses, isoelectric focusing gels were run without equilibration. Running gel was poured to 50-mm below the base of the notch. A 50-mm stacking gel was prepared following the same procedure as the standard SDS slab gel. IEF gel was loaded directly on top of the slab gels and the tracking dye, bromophenol blue, was added. The slab gels were mounted on the gel tank, and 2% SDS running buffer was put in the upper reservoir and regular running buffer in the lower reservoir. The gels were run at 20 mA for 20 min. The 2% SDS running buffer was then replaced with a standard running buffer and electrophoresis was continued until the tracking dye front reached the end of the gel.

Following electrophoresis, the gels were fixed in a 40% ethanol—7% acetic acid solution for 48 hr with four changes of fixative. Gels were stained and destained and electrophoretic patterns recorded as described. Molecular weights were determined by comparing relative mobilities of protein bands with those of standard proteins.

RESULTS & DISCUSSION

ALL EXPERIMENTS with 12 fish species were repeated at least twice; they all showed reproducible protein patterns. Further-

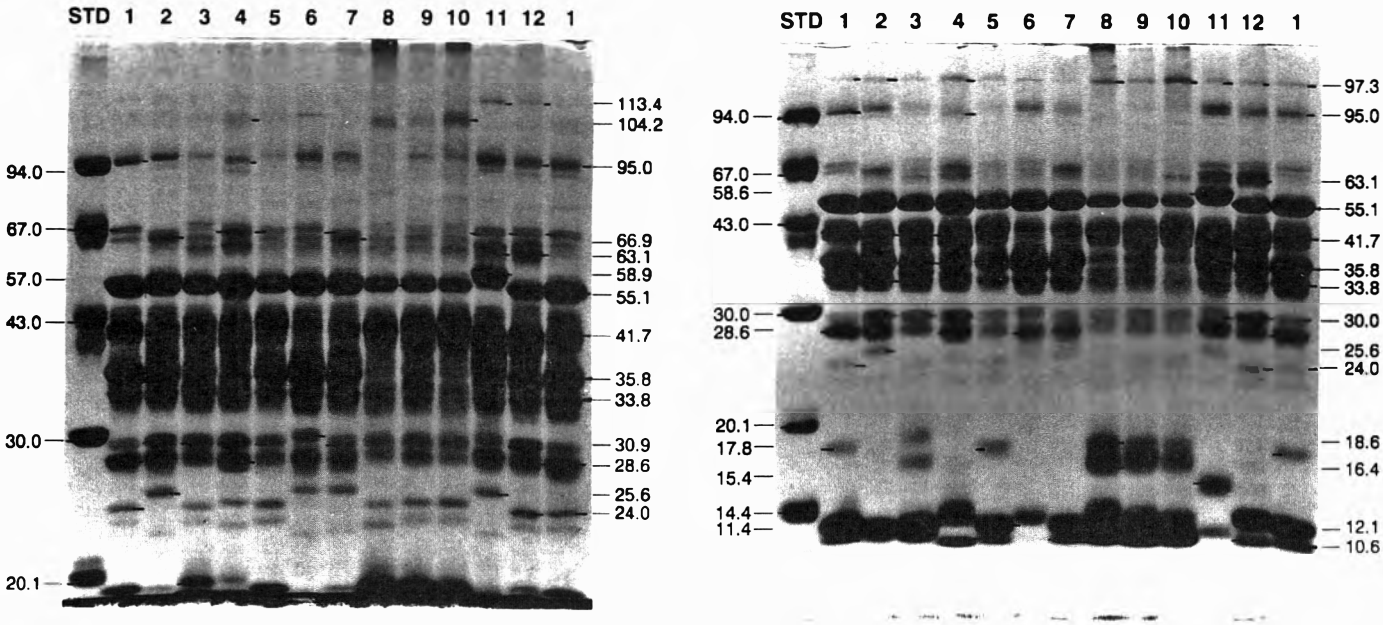

Fig. 2—Protein profiles of water-soluble muscle sarcoplasmic extracts from various fish samples on (A) 10%, and (B) 12.5% SDS-PAGE. Fish samples are (1) RS, (2) VS, (3) GS, (4) HF, (5) LS, (6) MS, (7) YS, (8) OS, (9) BS, (10) LL, (11) PG, and (12) WG. The numerical designations indicate molecular weight of the protein bands.

Table 1—Distribution of water-soluble sarcoplasmic proteins (with pIs) of 12 fish species on IEF gels using pH 3–10 and 4–6.5 ampholytes, and an ampholyte mixture of 20% pH 3–10 and 80% pH 4–6.5

Fish species	pH 3–10 Ampholytes	pH 4–6.5 Ampholyte	Ampholyte mixture
Red snapper (RS)	6.14, 5.98, 4.77, 4.52, 3.76, 3.48	6.54, 6.34, 6.21, 6.01, 5.25, 5.04, 4.98, 4.69, 4.49	7.26, 7.10, 7.05, 6.90. 6.69, 5.96, 5.76, 5.65, 5.36, 5.18
Vermilion snapper (VS)	6.14, 5.01, 4.77, 3.76, 3.48	6.54, 5.50, 5.25, 5.04, 4.69, 4.49	7.26, 7.10, 6.72, 6.18, 5.96, 5.36, 5.18
Gray snapper (GS)	6.14, 5.98, 5.90, 4.35, 3.38, 3.23	6.34, 6.21, 6.10, 4.98, 4.82, 4.69, 4.46, 4.35	7.26, 7.10, 6.90, 6.72, 5.76, 5.65, 5.49, 5.13, 5.02
Hogfish (HF)	6.14, 5.98, 5.90, 3.90, 3.63	6.34, 6.21, 6.10, 6.01, 4.82, 4.69, 4.60	7.26, 7.10, 7.05, 6.90, 6.72, 5.57, 5.44, 5.36, 5.26
Lane snapper (LS)	6.14, 4.61, 3.48	6.34, 5.12, 4.82, 4.53, 4.49	7.10, 5.79, 5.59, 5.22, 5.18
Muttcn snapper (MS)	4.77	6.21, 5.30, 5.04, 4.60	7.10, 5.96, 5.76, 5.26
Yellowtail snapper (YS)	4.61, 3.63	6.21, 5.04, 4.60, 4.49	6.90, 5.76, 5.26, 5.13
Onespot snapper (OS)	6.14, 5.98, 5.85, 4.77, 3.90, 3.48, 3.38	6.49, 6.21, 6.10, 4.75, 4.52, 4.49, 4.35	7.26, 7.10, 6.90, 6.69, 5.49, 5.47, 5.18, 5.08
Blackspot snapper (BS)	6.14, 5.98, 5.85, 4.77, 4.14, 3.48, 3.38, 3.26	6.49, 6.21, 6.10, 6.01, 4.80, 4.49, 4.29	7.26, 7.10, 6.90, 6.69, 5.57, 5.53, 5.18, 5.08, 5.02
Madras snapper (LL)	6.14, 5.98, 5.85, 3.85, 3.76, 3.63, 3.55, 3.38	4.73, 4.69, 4.52	7.26, 7.10, 5.47, 5.36, 5.26, 5.11
Pink porgy (PG)	5.01, 4.52	5.45, 5.01	7.05, 6.18, 6.10, 5.76
White grunt (WG)	6.14, 5.98, 5.85, 4.35, 3.95, 3.76, 3.38	4.77, 4.35	7.26, 7.10, 5.65, 5.53, 5.49, 5.36, 5.11

more, the IEF and SDS-PAGE protein profiles conducted by different operators following the same protocol procedures, all showed reproducible results. Red snapper from the Texas and Florida coasts off the Gulf of Mexico showed similar gel electrophoretic protein patterns as authentic red snapper (data not shown). No difference in protein patterns was observed between red snappers from these two different locations.

IEF gels

A broad range pH 3–10 ampholyte was employed in order to obtain more protein bands for species comparison. Protein bands with pI values between 3.23 and 5.5 were well separated and readily recognizable (Fig. 1). Each of the 12 species demonstrated unique protein profiles. They each had species-specific proteins, but, also had some proteins found in patterns of other species (Fig. 1 and Table 1). By comparing protein profiles on the pH 3–10 IEF gel, RS could be identified.

Note that proteins from each species having pI values of 5.8–6.8 were stacked together on the pH 3–10 IEF gel, while those with pI $>$ 6.8 were blurred. This made protein identification difficult. Ampholytes at different pH ranges of 4–6.5, 6–8, and 5–6, as well as an ampholyte mixture containing 20% pH 3–10 and 80% pH 4–6.5 were also used to separate proteins of designated pI ranges on IEF gels for species identification. Proteins with pI values of 4.35–5.50 in each fish species were well separated using the pH 4–6.5 IEF gel (Table 1). Furthermore, proteins with pI values of 5.50–6.34 were better separated in this IEF gel than the pH 3–10 gel, although proteins with pI values $>$ 6.34 were not well separated (data not shown). Fish samples

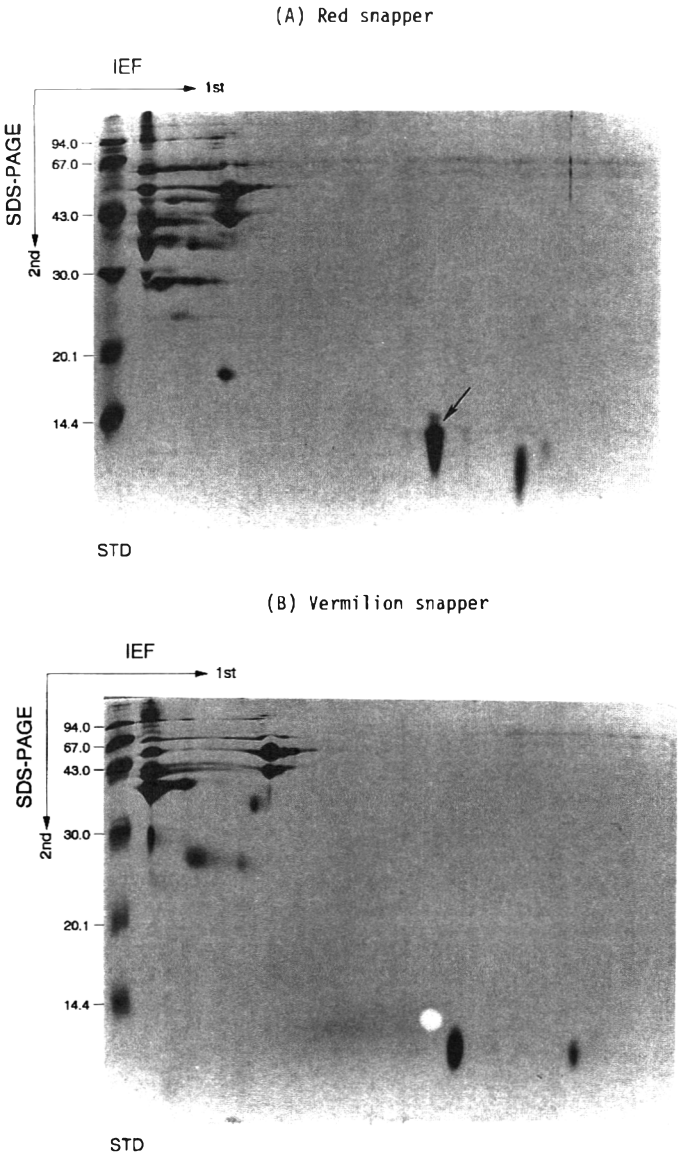

Fig. 3—Two-dimensional gel electrophoretic patterns of the water-soluble muscle sarcoplasmic protein extracts from (A) RS and (B) VS. Top left corner represents pH 7.7 of the gel and top right corner pH 4.8. The protein standards (STD) are also included.

of different species contained some proteins with the same pI values. They also showed species-specific proteins. The specific protein profile of each fish species in the pH 4–6.5 IEF gel could thus be used for species identification.

Fish samples also showed species-specific protein patterns on the pH 6–8 and pH 5–6 IEF gels useful for identification. Proteins with pI values of 6.56 to 8.0 were better separated in pH 6–8 IEF gel than in pH 3–10 gel, although those with pI 6.8 to 7.6 stacked together (data not shown). The pI 7.30 protein was not found in YS and PG, the pI 6.95 protein was not in PG, and the pI 6.85 protein was not in VS, MS, and PG. The pI 6.79 protein was found in RS, GS, PG, and HF. Only GS had the pI 6.73 protein, and WG had the pI 6.60 and 6.56 proteins. The pH 5–6 IEF gel also showed similar protein patterns for the 12 fish species as the pH 4–6.5 gel (data not shown). Some protein bands in the pH 4–6.5 or 3–10 IEF gel, thought to contain only one protein, pI 5.0 to 6.0, were shown to consist of two proteins in the pH 5–6 IEF gel due to better separation (data not shown). Proteins with pI values < 5.0 were not well separated in the pH 5–6 IEF gel compared to pH 4–6.5 gel.

Better protein separation occurred in IEF gels containing a 20% pH 3–10 and 80% pH 4–6.5 ampholyte mixture (Wei et al., 1990). Using this system, many proteins appeared in the pH range 5.0 to 7.7 (Table 1). The patterns of fish proteins with pI 5–6.5 were similar to those on the pH 4–6.5 IEF gel (data not shown). Many proteins with pI > 7.0, that were not found in the pH 4–6.5 gel, appeared in this gel containing the ampholyte mixture. These proteins clustered in a narrow area at pH 7.0–7.7 and were not well separated. Each fish species displayed an individual protein pattern. Because more protein bands appeared in the pH range 5.0–7.7, that system may be more effective than the three previously mentioned IEF gels for species identification.

SDS gels

The 7.5, 10.0, 12.5 and 15.0% polyacrylamide gels were used to separate water-soluble sarcoplasmic proteins from 12 fish samples. Distinctive protein profiles were noted with each species using the 10% SDS gel, although patterns for GS, LS, OS, BS, and LL were similar (Fig. 2A). Proteins with MW 22.0 to 31.0 kD and 55.1 to 113.4 kD were well separated, while those major proteins with MW between 31.0 and 55.1 kD clustered together. The 104.2 kD protein was in HF, OS, BS, and LL; the 67.0 kD protein was in RS, HF, LS, YS, PG and WG; and the 58.9 kD protein only in PG. Except for OR, all fish species contained the 95.0 kD protein. The 55.1 kD protein was detected in RS, VS, GS, HF, MS, YS and WG; the 25.6 kD protein was in VS, MS, YS, and PG; and the 24.0 kD protein was in RS, GS, LS, BS, LL, and WG.

The protein patterns of the 12 fish species shown in the top ⅔ of the 12.5% gel were, in general, similar to those with the 10% gel. However, those proteins having MW between 15 and 24 kD were well separated in the 12.5% SDS gel (Fig. 2B). OS, BS and LL had similar protein patterns, while each remaining fish species had its own distinctive profile useful for identification. An 18.6 kD protein was found in RS, LS, OS, BS, and LL; the 17.8 kD protein was in RS, VS, LS, OS, BS, LL, and PG; and the 12.1 kD protein was in RS, GS, LS, YS, OS, BS, and LL. Only PG was found to have the 15.4 kD protein.

Proteins with MW < 30 kD were well separated in the 15% SDS gel (data not shown). The protein patterns were similar to those of the 12.5% gel except that the distance between protein bands in the 15% gel was closer. The 15% gel had smaller pores to retard protein movement. The 13.6 kD protein was found in RS, HF, LS, MS, YS, OS, BS, LL, and WG, and the 13.2 kD protein was in VS, GS, LS, YS, OS, BS, LL, and WG. The 12.7 and 15.2 kD proteins were found in HF and PG, respectively. Each fish species showed distinctive protein profiles.

Proteins with larger molecular weights were better separated in 7.5% SDS gel; those with MW 55.1 to 95.0 kD were well separated, while those with MW < 55.1 kD stacked together (data not shown). The 95.0 kD protein was in RS, HF, PG, and WG; the 66.9 kD protein was in RS, HF, YS, PG, and WG; and the 65.6 kD protein was in YS and PG. The 63.1 kD protein was in GS, LL, PG, and WG. The 58.9 kD protein was only in PG, while the 55.1 kD protein was missing only in PG. Protein profiles from 7.5% SDS gel were not as effective as those from 10, 12.5 or 15% gels for species identification.

Two-dimensional gel electrophoresis

The protein patterns of seven authentic fish samples (RS, VS, GS, HF, LS, MS, and YS) in the 2-D gel system were compared for differences in protein pI values and molecular weights; only patterns of RS and VS are shown (Fig. 3). More protein spots were obtained for each sample in the 2-D gel than in the IEF or SDS-PAGE gel. Many proteins with pI < 6.0 on the IEF gels did not appear in the 2-D gels. Proteins with pI values $>$ 6.5, not well separated in the mixed IEF gel (first dimension), were separated in the second dimension. Some such bands, especially those with greater pI values, which appeared to contain only one protein band in the IEF gel, showed more than one protein after the second dimension (Fig. 3). Since each of the

seven authentic fish samples showed its own unique protein pattern, the 2-D gel could also be used for fish species identification.

Alternative electrophoretic methods could thus be used for regulatory purposes to differentiate red snapper from closely related fish species, including VS, GS, LS, MS, YS, PG, WG, and HF, often used as illegal substitutes for RS. Hsieh et al. (1993) used thin-layer IEF to check 81 labeled as RS fillet samples collected by food inspectors from retail supermarkets and fish dealers across Florida between 1988 and 1992 and found that only 24 (30%) samples were true RS. Scarlet snapper (*Lutjanus sanguineus*), GS, LS, malabar snapper (*Lutjanus malabaricus*), MS, VS, and YS were used as substitutes for RS and accounted for 58% mislabeling. The combined use of IEF, SDS-PAGE and 2-D electrophoresis would also help resolve the polymorphism problem encountered by Lundstrom (1981).

REFERENCES

An, H., Marshall, M.R., Otwell, W.S., and Wei, C.I. 1988. Electrophoretic identification of raw and cooked shrimp using various protein extraction systems. J. Food Sci. 53: 313–318.

An, H., Marshall, M.R., Otwell, W.S., and Wei, C.I. 1989. Species identification of raw and cooked shrimp by urea gel isoelectric focusing technique. J. Food Sci. 54: 233–236, 257.

AOAC. 1990. *Official Method of Analysis*, 15th ed., p. 883–889. Association of Official Analytical Chemists, Washington, DC.

FAO Species Catalogue. 1985. *Snappers of the World.* An Annotated and Illustrated Catalogue of *Lutjanid* Species Known to Date. FAO Fisheries Synopsis, Vol. 6, No. 125 (6), p. 3. Food and Agriculture Organization of the United Nations, Rome.

FDA. 1980. Fish and Seafood. Guide 7108.21. Ch. 8, *Food and Drug Administration Compliance Policy Guides*, FDA, Washington, DC.

Hsieh, Y.H.P., Kirsch, P.F., and Woodward, B.B. 1989. Species identification of retail snapper fillets in Florida. Proceedings in Tropical and Subtropical Fisheries Technological Society of the Americas, 1989, p. 218–224. Florida Sea Grant College Program, University of Florida, Gainesville, FL.

Hsieh, Y.P., Woodward, B.B., and Walden, A.L. 1993. Species identification of retail snapper fillets in Florida. Abstract No. 236, 1993 Annual Meeting of the Institute of Food Technologists, July 10–14, Chicago.

Keung, M.W., Ditlow, C.C., and Vallee, B.L. 1985. Identification of human alcohol dehydrogenases isozymes by disc polyacrylamide gel electrophoresis in 7 M urea. Anal. Biochem. 151: 92–96.

Låås, T., Olsson, I., and Söderberg, L. 1980. High-voltage isoelectric focusing with pharmalyte: field strength and temperature distribution, zone sharpening, isoelectric spectra, and pI determinations. Anal. Biochem. 101: 449–461.

Laemmli, U.K. 1970. Cleavage of structure proteins during the assembly of the head of bacteriophage T4. Nature 227: 680–685.

Lowry, O.H., Rosebrough, N.J., Farr, A.L., and Randall, R.J. 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. 193: 265–275.

Lundstrom, R.C. 1979. Fish species identification by thin layer isoelectric focusing. J. Assoc. Off. Anal. Chem. 62: 624–629.

Lundstrom, R.C. 1980. Fish species identification by thin layer polyacrylamide gel isoelectric focusing: collaborative study. J. Assoc. Off. Anal. Chem. 63: 69–73.

Lundstrom, R.C. 1981. Fish species identification by isoelectric focusing: sarcoplasmic protein polymorphism in monkfish (*Laphius americans*). J. Assoc. Off. Anal. Chem. 64: 32–37.

Lundstrom, R.C. 1983a. Fish species identification by agarose gel isoelectric focusing: Collaborative study. J. Assoc. Off. Anal. Chem. 66: 123–127.

Lundstrom, R.C. 1983b. Identification of Pacific rockfish (*Sebastes*) species by isoelectric focusing. J. Assoc. Off. Anal. Chem. 66: 974–980.

Melvin, M. 1987. *Electrophoresis*, p. 40. John Wiley and Sons, New York.

O'Farrell, P.H. 1975. High resolution two-dimensional electrophoresis of proteins. J. Biol. Chem. 250: 4007–4021.

Weber, K., Pringle, J.R., and Osborn, M. 1972. Measurement of molecular weights by electrophoresis on SDS-acrylamide gel. Meth. Enzymol. 26: 3–27.

Wei, C.I., An, H., Chen, J., and Marshall, M.R. 1990. Use of a modified urea gel isoelectric focusing method for species identification of raw or broiled white, pink, and rock shrimp. J. Food Biochem. 14: 91–102.

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lation or denaturation of plasma protein on heat treatment would be suppressed by conjugation with GM.

The emulsifying properties of the plasma protein-GM complex (complex 2) and native plasma protein were also compared (Table 2) before and after heat treatment at 80°C for 30 min in a high-salt buffer (0.066 M sodium phosphate buffer, pH 7.4, containing 0.2M NaCl). The emulsifying properties of native plasma protein decreased greatly after heat treatment, possibly because that sample contained considerable insoluble precipitates. However, there were no deteriorative effects on emulsifying properties of the plasma protein-GM complex after heat treatment. Thus, plasma protein-GM complex seemed to be stable to heat. Such heat stability would be favorable to heat pasteurization for food applications.

CONCLUSIONS

PLASMA PROTEIN-GM COMPLEX had excellent emulsifying properties compared to native plasma protein, and its heat stability was notably enhanced. The plasma protein-GM complex showed more stable emulsifying properties than commercial emulsifiers under various conditions.

REFERENCES

Dubois, M., Gilles, K.A., Hamilton, J.K., Rebers, P.A., and Smith, F. 1956. Colorimetric method for determination of sugars and related substances. Anal. Chem. 28: 350–356.

Kato, Y., Matsuda, T., Kato, N., and Nakamura, R. 1989. Maillard reaction of disaccharides with protein: suppressive effect of nonreducing end pyranoside groups on browning and protein polymerization. J. Agric. Food Chem. 37: 1077–1081.

Kato, A., Sasaki, Y., Furuta, R., and Kobayashi, K. 1990. Functional properties-polysaccharide conjugate prepared by controlled dry-heating of ovalbumin-dextran mixtures. Agric. Biol. Chem. 54: 107–112.

Kitabatake, N., Cuq, J.L., and Cheftel, J.C. 1985. Covalent binding of glycosyl residues to β-lactoglobulin: effects on solubility and heat stability. J. Agric. Food Chem. 33: 125–130.

Laemmli, U.K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) 227: 680–685.

Lee, H.S., Sen, L.C., Clifford, A.J., Whitaker, J.R., and Feeney, R.E. 1979. Preparation and nutritional properties of caseins covalently modified with sugars: reductive alkylation of lysines with glucose, fructose, or lactose. J. Agric. Food Chem. 27: 1094–1098.

Lowry, O.H., Rosebrough, N.J., Farr, A.L., and Randall, R.J. 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. 193: 265–275.

Marsh, J.W., Denis, J., and Wriston, J.C. 1977. Glycosylation of Escherichia coli L-asparaginase. J. Biol. Chem. 252: 7678–7684.

Marshall, J.J. and Rabinowitz, M.L. 1976. Preparation and characterization of a dextran-trypsin conjugate. J. Biol. Chem. 251: 1081–1087.

Matsudomi, N., Tsujimoto, T., Kato, A., and Kobayashi, K. 1994. Emulsifying and bactericidal properties of a protamine-galactomannan conjugate prepared by dry heating. J. Food Sci. 59: 428–431.

Miller, G.L. 1959. Protein determination for large numbers of samples. Anal. Chem. 31: 964.

Nakamura, S., Kato, A., and Kobayashi, K. 1992. Bifunctional lysozyme-galactomannan conjugate having excellent emulsifying properties and bactericidal effect. J. Agric. Food Chem. 40: 735–739.

Pearce, K.M. and Kinsella, J.E. 1978. Emulsifying properties of proteins: Evaluation of a turbidimetric technique. J. Agric. Food Chem. 26: 716–723.

Saito, M., Ichikawa, N., and Taira, H. 1988. Fractionation and emulsifying properties of plasma proteins. Agric. Biol. Chem. 52: 2831–2836.

Saito, M. and Taira, H. 1987. Heat denaturation and emulsifying properties of plasma protein. Agric. Biol. Chem. 51: 2787–2792.

Shimada, K. and Matsushita, S. 1981. Thermal coagulation of bovine serum albumin. Agric. Biol. Chem. 45: 1945–1952.

Tybor, P.T., Dill, C.V., and Landmann, W.A. 1975. Functional properties of proteins isolated from bovine blood by a continuous pilot process. J. Food Sci. 40: 155–159.

Yamamoto, T., Yamamoto, S., Miyahara, I., Matsumura, Y., Hirata, A., and Kin, M. 1990. Isolation of a β-mannan hydrolyzing enzyme and hydrolysis of guar gum by the enzyme isolated. Denpun Kagaku 37: 99–105.

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Frozen Storage Quality of Rainbow Trout (*Oncorhynchus mykiss*) as Affected by Oxygen, Illumination, and Fillet Pigment

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ABSTRACT

We investigated the influence of packaging materials with high, medium and low oxygen transmission rates (OTR) on the development of 2-thiobarbituric acid-reactive substances (TBARS) and astaxanthin retention in rainbow trout fillets. Three different levels of astaxanthin, during dark or illuminated frozen storage were studied. Samples were analyzed after 17, 29 and 36 wk frozen storage. Rancidity developed more rapidly in packages with high OTR. This effect was more pronounced in illuminated samples, also verified by sensory evaluation. Fillets with highest astaxanthin content reached maximum TBARS after 29 wk. The two less pigmented sample groups reached maximum after 17 wk frozen storage, indicating antioxidative effects of astaxanthin.

Key Words: lipid oxidation, frozen storage, rainbow trout, astaxanthin, antioxidant

INTRODUCTION

DETERIORATION OF FOOD is often caused by the production of fatty acid hydroperoxides, precursors of rancid flavor substances. Terao (1989) and Miki (1991) demonstrated that astaxanthin and canthaxanthin were more efficient singlet oxygen ($^1O_2$) quenchers and radical scavengers than β,β-carotene and zeaxanthin in preventing peroxidation of lipids. Carotenoids are very efficient antioxidants under low oxygen partial pressure (Burton and Ingold, 1984), and addition of β,β-carotene to vegetable oils reduces their peroxidation rate (Fakourelis et al. 1987; Jung and Min, 1991). The influence of light can induce unwanted changes in foods (Bekbölet, 1990), like off-flavor, loss of vitamin activity, photosensitized oxidation of lipids and oxidation of pigments (carotenoids and myoglobin) associated with loss of natural color. The antioxidant function of carotenoids has received considerable attention (Krinsky, 1989). Also, focus on protecting foods high in polyunsaturated fatty acids (PUFA) is increasing due to possible relations between lipid oxidation products and atherosclerosis (Kubow, 1993).

The photoprotective function of carotenoids in photosynthesizing or nonphotosynthesizing organisms is well documented (Kornhauser et al. 1989; Will and Scovel, 1989). The light energy absorbed by carotenoids is emitted by fluorescence and molecular vibrations (Bondarev et al., 1989; Gillbro and Cogdell, 1989; Truscott, 1990; Wasielewski et al., 1989). Several researchers have reported carotenoid photodegradation in food systems (Andersen et al., 1990; Carnevale et al., 1979, 1980; Christophersen et al., 1991; Jørgensen and Skibsted, 1990; Najar et al., 1988; Pesek and Warthesen, 1987, 1990; Pesek et al., 1990; Sattar et al., 1977). The light-initiated degradation of carotenoids is increased by the presence of photosensitizers, due to the production of $^1O_2$ (Glória et al., 1993; Fakourelis et al., 1987; Jensen et al., 1982). Carotenoids are $^1O_2$ quenchers (Hirayama et al., 1994; Murasecco-Suardi et al., 1988) and protect lipids from photoperoxidation by acting as preferred substrates (Iliou et al., 1992). β,β-Carotene also inhibits the cyclooxygenation of arachidonic acid (Halevy and Sklan, 1987).

Important parameters, potentially different in wild and farmed fish (reviewed by Haard, 1992 and Love, 1988) may influence several quality attributes of fish. Considerable variation in lipid oxidation may occur within species (Erickson, 1993). Information is available on pigmentation of rainbow trout (Storebakken and No, 1992) and salmonids in general (Torrissen et al., 1989), but very little on coloration of salmonid products. Radiant flux density of UV light is more important than OTR of packaging materials in astaxanthin degradation in rainbow trout steaks during frozen storage (Christophersen et al., 1992). At present consumers seem to prefer light pink smoked salmon (Gormley, 1992). Several processing conditions affect sensory qualities of salmonid fish. Longer heat processing may result in darker color of rainbow trout products due to browning reactions (Chia et al., 1983). Andersen et al. (1990) showed that steaks of farmed rainbow trout (*Oncorhynchus mykiss*) with 9.1 mg astaxanthin/kg produced less TBARS than steaks of wild Atlantic salmon (*Salmo salar*) containing 4.9 mg astaxanthin/kg. They suggested radical scavenging effects of astaxanthin may explain this observation. However, the fatty acid content and composition of wild and farmed Atlantic salmon may vary significantly, depending on fatty acid sources of their feed (Cronin et al., 1991; Polvi and Ackman, 1992), and could possibly affect the rate of peroxidation. Large variations in carotenoid content occur in the flesh and color characteristics, between sexes and among individuals within sex in rainbow trout given the same feed (Röpke, 1988; Choubert et al. 1987; Blanc and Choubert, 1985). Differences also persist after cooking and smoke-curing processes (Choubert et al., 1992). Longitudinal variation in salmonid carotenoid content and red color, the caudal part containing up to 30–40% more carotenoids, has been reported (No and Storebakken, 1991; Christiansen and Wallace, 1988). Also there is a radial increase in pigmentation towards the backbone (McCallum et al., 1987). Therefore, whole fillets were used in our experiments.

Our objective was to compare astaxanthin content, TBARS and sensory attributes of fillets of rainbow trout given 3 different levels of astaxanthin in the feed, during frozen storage in an illuminated freezer cabinet or in darkness, packaged in 3 different materials with different OTRs, in order to evaluate antioxidant effectiveness of astaxanthin.

MATERIALS & METHODS

Feeding and rearing

Rainbow trout (*Oncorhynchus mykiss* Walbaum) with initial weight 130g, previously given a diet without astaxanthin supplementation, was divided into 3 groups. They were given an extruded diet with a common basic composition (Table 1) either not supplemented, or supplemented with 20 or 70 mg astaxanthin/kg dry feed ingredients, in order to produce three groups of rainbow trout with different contents of astaxanthin. Astaxanthin ("Carophyll Pink," 8%; Hoffmann-La Roche, Basel, Switzerland) was added as stabilized water-dispersible gelatin beadlets. The diets contained 8.1 (level 1), 28.1 (level 2) and 81.2 (level 3) mg astaxanthin/kg. Astaxanthin content in the feeds was determined by the method of Keller (1988). The three groups were fed these diets for 28 wk and reached a final weight of 900g. The trout were farmed by BP Nutrition ARC, Lerang Research Station, Jørpeland, Norway. The ex-

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Table 1—Basic formulation of the feed

Ingredients (g/kg)	
Fish meal (Norse LT-90)[a]	25.0
Fish meal (Norse Mink)[a]	32.0
NorSalmOil[a]	15.4
Wheat, extruded	21.5
Vitamins, minerals,[b] betaine concentrate[c]	6.1

[a] Norwegian Herring Meal and Oil Industries, Bergen, Norway.
[b] Proprietary formulation.
[c] Finnstim, Finnsurgar, Finland.

perimental design is summarized in Fig. 1. Two fish per treatment were analyzed at each sampling event.

Packaging and storage

Thirty specimens from each of the three groups of rainbow trout were filleted and deskinned. The head and tail were removed by cutting fillets just in front of the anal fin and half-way between the head and the front of the back fin. The right fillets from each fish were analyzed as soon as possible after filleting. The left fillets were divided into two equal sized pieces of about 150g by a transversal cut and packaged separately. One piece was for chemical analyses, the other for sensory evaluation. The samples were packaged and frozen as quickly as possible the same day the fish were slaughtered.

Three different packaging materials were used. All samples were stored at −18°C in an illuminated freezer cabinet. The light source was the ambient light from fluorescent lamps (TLD 58W/84, Philips) in a hall used daily for fish processing. The samples were either wrapped in polyethylene (PE) bags or vacuumed in PEA vacuum bags (polyethylene/polyamide, thickness 100 μm + 30 μm; oxygen transmission rate (OTR) 15 $cm^3/(m^2 \times 24$ h atm) at 23°C and 50% relative humidity). Air was allowed to flow freely into the unsealed PE-bags, in order to avoid oxygen-limitation. Identical packaging materials were employed for the frozen storage experiment (−18°C) in a dark cabinet, in addition to groups vacuumed in air-tight, non-transparent aluminum-laminated vacuum bags (polyetylene-terphthalate/aluminum/polypropylene).

Analytical methods

Percentage dry matter of fillets in different packaging materials was determined after dehydration at 105°C for 24 hr. Results were corrected for weight losses observed during frozen storage. Total lipid content of the fillets was determined after evaporation of the chloroform phase according to the method of Bligh and Dyer (1959). Pooled samples of two fish from each group were used. Final total fat content was 7.2 ± 0.2 (% w/w).

Distribution of fatty acids

The distribution of fatty acids was determined by gas chromatography (GC) of corresponding fatty acid methyl esters after alkaline hydrolysis of total lipid samples. Fatty acid methyl esters were prepared with BF_3 as catalyst. Samples were analyzed using a Shimadzu GC 14-A equipped with a Shimadzu AOC 14 auto-injector and a flame ionization detector. A DB-23 (50% (cyanopropyl)-methylpolysiloxane) fused silica column (length 30 m, i.d. 0.25 mm; J&W Scientific, Folsom, CA) was employed for chromatographic separation. Helium pressure was 1.2 kg/cm^2 and an injector temperature of 230°C and a detector temperature of 280°C were used. Initial oven temperature was 190°C (10 min) followed by an increase of 3°C/min for 8 min. The chromatograms were processed with Shimadzu Class LC 10 software and quantification was based on peak area. The fatty acid distribution of total fat was determined as a mean of two specimens from each diet treatment. No statistically significant differences were observed. The distribution of fatty acids was (% of total fatty acids (±SEM), mean of six fish): (14:0) 4.7 ± 0.3; (16:0) 15.7 ± 1.3; (18:0) 2.6 ± 0.2; (16:1) 6.9 ± 0.3; (18:1) 19.2 ± 0.6; (20:1) 8.4 ± 1.3; (22:1) 7.9 ± 1.0; (18:3, $n-3$) 1.2 ± 0.1; (18:4, $n-3$) 1.5 ± 0.1; (20:3, $n-3$) 0.12 ± 0.01; (20:4, $n-3$) 1.3 ± 0.1; (20:5, $n-3$) 0.30 ± 0.01; (21:5, $n-3$) 0.3 ± 0.3; (22:5, $n-3$) 1.6 ± 0.2; (22:6, $n-3$ and 24:0) 13.2 ± 1.1; (18:2, $n-6$) 4.6 ± 0.5; (20:2, $n-6$) 0.31 ± 0.02; (20:3, $n-6$) 0.14 ± 0.04; (20:4, $n-6$) 0.44 ± 0.05; (22:5, $n-6$) 0.18 ± 0.02 and others ca. 4.8.

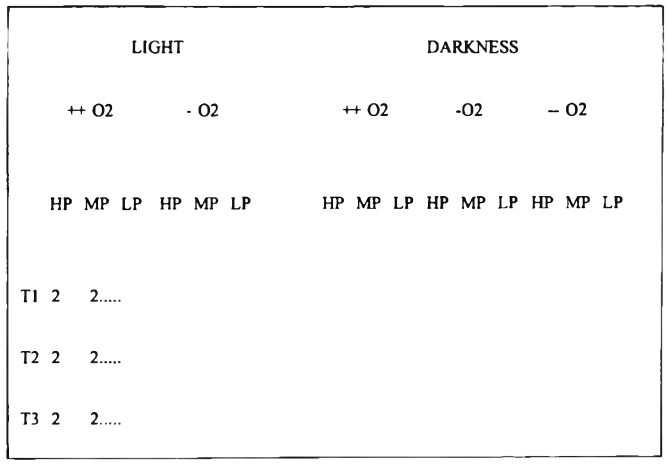

Fig. 1—Schematic of experimental design for packaging and illumination treatments of the different dietary groups, and sampling schedule. $++O_2$, $-O_2$, and $--O_2$ designates low, medium and high O_2-barrier, respectively. HP, MP and LP designate high, medium, and low pigmented trout fillets, respectively. T1---T3 indicates 17, 29 and 36 wk storage time, respectively, before sampling.

Carotenoids

Total carotenoid content in trout fillets was determined spectrophotometrically after extraction (2 × 50 mL) of the homogenized mince (10g) with a mixer (Ystral, Dottingen; 30 sec). The extracts were combined and centrifuged (10 min, 5000 rpm) and the volume adjusted to 100 mL with acetone. An aliquot was filtered through Whatman filter paper (black band) and the absorption measured at 472 nm. The amount of astaxanthin was determined by employing $E_{1\%,1\ cm} = 1900$. Duplicate analyses were performed on all fillets, and results are presented as means of two fish from each treatment. Carotenoid retention was calculated as the percentage of total carotenoid in a stored fillet of one fish, divided by the total carotenoid amount in the other fillet at the beginning of the experiment.

2-Thiobarbituric acid reactive substances (TBARS)

TBARS were determined spectrophotometrically according to Vyncke (1970). Accurately weighed samples (25g or less with high TBARS values) were extracted with a trichloroacetic acid (TCA) solution (7.5% TCA in water added 0.1% propyl gallate and EDTA, respectively) with a mixer (3 × 1 min). An aliquot (5 mL) of the filtered extract was reacted with a solution of TBA in 7.5% TCA (0.02M, 5 mL) in a sealed glass vial in a boiling water bath for 40 min. After completion of the reaction, samples were allowed to cool in cold water, and mixtures were centrifuged (5000 rpm, 10 min). The absorption was measured in a Beckman Model 35 spectrophotometer at 538 nm, with a 1:1 mixture of TCA extract and water as a reference. TBARS values were determined by comparison with those obtained by reaction of known amounts of 1,1,3,3-tetraethoxypropane with TBA. Duplicate analyses were performed on all fillets and TBARS are expressed in units mg of malondialdehyde/kg.

Sensory evaluation

Informal sensory evaluation of storage stability was mainly determined as rancidity on both thawed and steamed samples. A descriptive test (nine-graded scale) was used, with an in-house trained three-member panel, all experienced to evaluate rancid taste and smell. Samples were thawed at 4°C overnight, placed on a plate and evaluated. Samples were re-coded, vacuum-packaged, steamed for 20 min and re-evaluated. The evaluation scale was supplemented by comments when necessary.

Statistical analysis

Data were subjected to analysis of variance using a General Linear Model (GLM) procedure of the Statistical Analysis System package (SAS Institute, Inc., 1992) to study the effects of light, packaging material and pigment level on pigment retention and TBARS. Statistical significance was indicated for $P < 0.001$ (***), 0.01 (**) and 0.05 (*).

Comparisons between treatments were made by Tukey tests where appropriate.

RESULTS

THE INITIAL TOTAL CAROTENOID CONTENT in the flesh of the three groups (mean of 30 fish in each group) were 5.9 ± 0.8, 8.2 ± 1.4 and 14.8 ± 2.5 mg/kg for the groups supplemented with 8.1, 28.1 and 81.2 mg astaxanthin/kg dry feed, respectively. The astaxanthin levels in diets correlated significantly with the total amounts of astaxanthin deposited in the muscle tissue throughout the experiment ($p < 0.001$). The overall astaxanthin retention for all groups combined was 92, 83 and 79%, after 17, 29 and 36 wk frozen storage, respectively. This was significantly lower than the initial astaxanthin content. The initial TBARS was 0.26 ± 0.11 mg malondialdehyde/kg. No statistically significant difference in distribution of individual fatty acids was observed during the experiment (results not shown).

Least-square means for TBARS (Table 2) and astaxanthin retention (Table 3) were compared for different illuminations, packaging material and dietary astaxanthin levels. Frozen storage in light or packaging materials with high OTR resulted in elevated TBARS with $p < 0.001$ throughout the experiment, except for the illumination treatment after the shortest storage period ($p < 0.01$). These treatments also tended to give lowest astaxanthin retentions. The TBARS of the group with the highest dietary astaxanthin level did not reach its highest level until 29 wk of frozen storage, while the two lower pigmented groups apparently had reached their highest levels after 17 wk frozen storage. There was a tendency for a high dietary astaxanthin level to give higher TBARS ($p < 0.05$), but only after 29 and 36 wk storage. Conversely, a tendency towards higher astaxanthin retention for the lower dietary astaxanthin levels was only significant ($p < 0.05$) after 17 wk frozen storage.

Least-square means of TBARS and astaxanthin retention were compared for the different combinations of illumination, packaging materials and dietary astaxanthin levels. There was a tendency for higher dietary astaxanthin levels to result in higher TBARS (results not presented). There was little consistency in the influence of dietary astaxanthin level on astaxanthin retention, and considerable random variation within treatments.

Combination effects between packaging material and dietary astaxanthin levels were significant ($p < 0.05$) for TBARS throughout frozen storage. Combination effects between illumination and packaging material, and illumination and dietary astaxanthin level only were significant ($p < 0.05$) after 17 and 36 wk frozen storage. Combination effects between illumination and dietary astaxanthin level, and packaging material and dietary astaxanthin level were significant ($p < 0.05$) for astaxanthin retention after 17 and 36 wk frozen storage. Packaging in vacuum bags in darkness reduced TBARS ($p < 0.001$) throughout frozen storage, compared to storage in light and PE-bags. The aluminum laminated bags gave the best protection against lipid oxidation, and were significantly better than dark storage in vacuum bags only after the first storage period. Light treatment seemed to accentuate TBARS production. Elevated astaxanthin retention was observed for packaging materials with lower OTR, increasing when stored in darkness.

In general, the fish were found by informal sensory test to be more rancid when steamed than when raw. The packaging material had a strong effect on rancid flavor development, the aluminum-laminated bags (lowest OTR) giving the best protection. In addition, fillets stored in darkness generally received better scores than those stored in light. However, no trend was observed regarding the dietary astaxanthin level. The fillets stored in either vacuum bags or aluminum-laminated bags in darkness retained a highly acceptable quality throughout frozen storage, while those wrapped in PE-bags were unacceptable by the end of storage.

Table 2—Least-square means for development of TBARS (mg malonaldehyde/kg) for rainbow trout fillets treated with different illumination, packaging materials, and dietary astaxanthin levels[d] during frozen storage

	Storage time (wk)		
	17	29	36
Illumination			
1	2.29	3.68	2.79
2	2.89	2.03	1.50
Eff[e]	**	***	***
Packaging			
1	3.75[a]	4.17[a]	3.13[a]
2	1.43[b]	1.54[b]	1.67[b]
3	0.69[b]	0.40[c]	0.63[b]
Eff[e]	***	ab (***), bc (**)	***
Level			
1	2.20[a]	3.46[a]	2.54[a]
2	3.03[b]	2.68[b]	1.71[b]
3	2.54[a]	2.51[b]	2.19[a]
Eff[e]	*	*	*

[d] Treatment: Illum. 1 = light, 2 = darkness and Packaging 1, 2 and 3 are the materials with high, medium and low OTR, respectively, and Level 1, 2 and 3 are high, medium and low levels of dietary astaxanthin respectively.

[e] Effect of treatment, levels of significance $p < 0.05$ (*), $p < 0.01$ (**), $p < 0.001$ (***). Different letters within one column indicate significant differences between means in a treatment.

Table 3—Least-square means for development of astaxanthin retention (%) for rainbow trout fillets treated with different illumination, packaging materials, and dietary astaxanthin levels[c] during frozen storage

	Storage time (wk)		
	17	29	36
Illumination			
1	88.1	79.0	74.6
2	88.2	87.3	76.2
Eff[d]	ns	ns	ns
Packaging			
1	85.4[a]	81.6	72.0[a]
2	91.0[a]	84.7	78.9[a]
3	107.2[b]	83.3	94.7[b]
Eff[d]	**	ns	*
Level			
1	83.4[a]	80.1	74.4
2	88.1[a]	81.6	73.6
3	95.0[b]	87.8	78.3
Eff[d]	*	ns	ns

[c] Treatment: Illum. 1 = light, 2 = darkness and Packaging 1, 2 and 3 are the materials with high, medium and low OTR, respectively, and Level 1, 2 and 3 are high, medium and low levels of dietary astaxanthin, respectively.

[d] Effect of treatment, levels of significance $p < 0.05$ (*), $p < 0.01$ (**), $p < 0.001$ (***) and not significant (ns). Different letters within one column indicate significant differences between means in a treatment.

DISCUSSION

ASTAXANTHIN DEPOSITION in fillets correlated highly with dietary astaxanthin level ($p < 0.0001$). At this size (about 130 g) rainbow trout has a rapidly increasing carotenoid deposition (Bjerkeng et al., 1992), known to correlate with growth rate (Torrissen et al., 1989; Bauvineau et al., 1993).

The fish fillets were stored at −18°C either in darkness or in an illuminated freezer cabinet in order to simulate conditions in the retail trade. The carotenoid loss rates we found were considerably less than those reported by Chen et al. (1984) and Pozo et al. (1988), although higher than those reported by No and Storebakken (1991) for vacuum-packaged rainbow trout fillets (skin on) stored in darkness. The relative stability of fatty acids seems to confirm the results reported by Polvi et al. (1991).

According to Andersen and Steinsholt (1992), frozen storage at decreasing temperatures between −13 and −35°C significantly increased color retention. Temperature was the single most important factor governing quality of Atlantic salmon flesh during frozen storage. Our results clearly demonstrated that the OTR of packaging materials, as well as illumination, affected

the development of rancidity and frozen storage life of salmonid fillets. This was also supported by informal sensory evaluation. This was in agreement with the results of Hwang and Regenstein (1988) showing the efficiency of vacuum-packaging in retarding oxidative rancidity during frozen storage of menhaden mince. The formation of TBARS was reduced ($p < 0.0001$) by packaging in materials with low OTR's, thus confirming earlier reports (Chen et al., 1984; Christophersen et al., 1992). Reduced formation of TBARS ($p < 0.001$) was observed in samples stored darkness and confirmed results of Jørgensen and Skibsted (1990) and Christophersen et al. (1991, 1992). Photodegradation of carotenoids depends on light wavelength, lower wavelengths resulting in more degradation (Jørgensen and Skibsted, 1990; Sattar et al., 1977). Packaging materials with UV-light absorbers or light-impermeable materials would therefore likely improve surface color retention of illuminated foods containing carotenoids.

Several researchers have presented evidence for a free radical mechanism of autocatalytic photodegradation of carotenoids and inhibition of photosensitized oxidation of PUFA by carotenoids (Camevale et al., 1979; Frankel, 1991). Carotenoids are known to influence production of flavor compounds in salmonids by favoring formation of 2-keto-3,5-octadienes over 2,4-heptadienals during the decomposition of *n*-7-hydroperoxides of PUFA (Josephson et al., 1991). Ingemansson et al. (1993), however, did not find any effect of astaxanthin supplementation on lipid stability to hydrolysis or oxidation. Their investigation compared white flesh of two groups of rainbow trout containing only 1.3 and 1.9 mg astaxanthin/kg, respectively (low-pigmented fish). The small difference in pigmentation between groups may account for their result. Similarly, Sigurgisladottir et al. (1994) found no antioxidant effect of astaxanthin on lipid stability or taste/texture in Atlantic salmon. In our experiment, rainbow trout fillets with highest astaxanthin level had less TBARS than the other groups after 17 wk frozen storage. The TBARS increased further until 29 wk, while the level in the two less-pigmented groups seemed to have reached maximum levels of TBARS after 17 wk frozen storage. This apparently conformed to a typical development of TBARS from oxidation of PUFA (Boyd et al., 1992). However, the differences in TBARS for trout given different dietary astaxanthin levels were not as pronounced as those reported by Andersen et al. (1990). They compared wild Atlantic salmon and pen-reared rainbow trout, but indicated the converse relationship. Intrinsic factors such as muscle vitamin E content (Waagbø et al., 1993), environmental acclimation (Sänger, 1993) as well as species differences in muscle structure may account for some differences in lipid stability.

Results for TBARS in illuminated fillets, especially those with limited oxygen, show that the lowest pigmented fillets had a more rapid increase in TBARS. This may be due to the singlet oxygen quenching effects of astaxanthin, especially the inhibition of the initial nonradical photoperoxidation steps involving singlet oxygen. There is also a concomitant delay of radical lipid peroxidation, as discussed by Miki (1991) and Iliou et al. (1992). Oxygen sensitivity of astaxanthin is also indicated by the lower retentions given for the high-dietary astaxanthin groups.

Turbot given high levels of oxidized fish oils in the diet had elevated TBARS in the flesh and reduced levels of the antioxidant vitamins C and E in the liver (Obach and Baudin Laurencin, 1992). This is probably true for salmonid species as well, emphasizing the importance of high-quality oils (presently about 30% of most commercial fish feeds). Erickson (1993) has shown that increasing TBARS and peroxides were accompanied by decreasing levels of α-tocopherol in the flesh mince of channel catfish during frozen storage. Dietary vitamin E can increase storage stability and sensory properties of fish fillets (Frigg et al., 1990; Waagbø et al., 1993) by preventing lipid oxidation. Our results suggest that astaxanthin may retard lipid peroxidation, especially in fillets subjected to illumination or packaging materials with high OTR. This confirms the early results of Lewis et al. (1958) who reported slightly higher flavor scores during frozen storage of turkeys given diets supplemented with xanthophylls.

Packaging treatment (OTR) had significant effects on astaxanthin retention. Light or initial astaxanthin level in the flesh did not influence astaxanthin retention significantly. However, note that the astaxanthin concentration was determined in whole fillet homogenates, possibly obscuring the photocatalyzed pigment loss occurring at the surface of the fillets (Christophersen et al., 1992).

CONCLUSIONS

ASTAXANTHIN has useful antioxidative effects during frozen storage of rainbow trout fillets, as well as production of desirable coloration. Proper packaging and storage conditions are important for lipid stability. Packaging materials that are good oxygen barriers inhibited development of rancidity. Modified atmosphere packaging with low oxygen content should increase color stability of frozen salmonid flesh. Prolonged exposure of salmonid flesh to illumination should be avoided due to the catalytic effect on lipid deterioration.

REFERENCES

Andersen, U.B. and Steinsholt, K. 1992. Deepfrozen salmon: Differences in quality after storage at different temperatures following different storage periods. Norw. J. Agric. Sci. 6: 211–215.

Andersen, H.J., Bertelsen, G., Christophersen, A.G., Ohlen, A., and Skibsted, L.H. 1990. Development of rancidity in salmonoid steaks during retail display. A comparison of practical storage life of wild salmon and farmed rainbow trout. Z. Lebensm. Unters. Forsch. 191: 119–122.

Bauvineau, C., Laroche, M., Heil, F., Dumont, J.-P., Cardinal, M., Cornet, J., Vallet, J.-L., Fauconneau, B., and Faure, A. 1993. Incidence de la vitesse de croissance sur les caracteristiques de la chair de la truite fario (*Salmo trutta*) elevee en mer. *Sci. Alim.* 13, 201–211.

Bekbölet, M. 1990. Light effects on food. J. Food Protect. 53: 430–440.

Bjerkeng, B., Storebakken, T., and Liaaen-Jensen, S. 1992. Pigmentation of rainbow trout from start feeding to sexual maturation. Aquaculture 108: 333–346.

Blanc, J.-M. and Choubert, G. 1985. Genetic variation of flesh colour in canthaxanthin fed rainbow trout. Génét. Sél. Evol. 17: 243–250.

Bligh, E.G. and Dyer, W.J. 1959. A rapid method of total lipid extraction and purification. Can. J. Biochem. Physiol. 37: 911–917.

Bondarev, S.L., Bachilo, S.M., Dvornikov, S.S., and Tikhomirov, S.A. 1989. $S_2 \rightarrow S_0$ fluorescence and transient $S_n \leftarrow S_1$ absorption of all-*trans*-β-carotene in solid and liquid solutions. J. Photochem. Photobiol. 46: 315–322.

Boyd, L.C., King, M.F., and Sheldon, B. 1992. A rapid method for determining the oxidation of n-3 fatty acids. J. Am. Oil Chem. Soc. 69: 325–330.

Burton, G.W. and Ingold, K.U. 1984. β-Carotene: An unusual type of lipid antioxidant. Science 224: 569–573.

Carnevale, J., Cole, E.R., and Cranx, G. 1979. Fluorescent light-catalyzed autoxidation of β-carotene. J. Agric. Food Chem. 27: 462–463.

Carnevale, J., Cole, E.R., and Crank, G. 1980. Photocatalyzed oxidation of paprika pigments. J. Agric. Food Chem. 28: 953–956.

Chen, H.-M., Meyers, S.P., Hardy, R.W., and Biede, S.L. 1984. Color stability of astaxanthin pigmented rainbow trout under various packaging conditions. J. Food Sci. 49: 1337–1340.

Chia, S.S., Baker, R.C., and Hotchkiss, J.H. 1983. Quality comparison of thermoprocessed fishery products in cans and retortable pouches. J. Food Chem. 48: 1521–1525, 1531.

Choubert, G., Guillou, A., and Fauconneau, B. 1987. Absorption and fate of labelled canthaxanthin 15,15'-$^3H_2$ in rainbow trout (*Salmo gairdneri* Rich.). Comp. Biochem. Physiol. 87A: 717–720.

Choubert, G., Blanc, J.-M., and Courvalin, C. 1982. Muscle carotenoid content and colour of farmed rainbow trout fed astaxanthin or canthaxanthin as affected by cooking and smoke-curing procedures. Int. J. Food Sci. Technol. 27: 277–284.

Christiansen, J.S. and Wallace, J.C. 1988. Deposition of canthaxanthin and muscle lipid in size groups of Arctic charr, *Salvelinus alpinus* (L.). Aquaculture 69: 69–78.

Christophersen, A.G., Jun, H., Jørgensen, K., and Skibsted, L.H. 1991. Photobleaching of astaxanthin and canthaxanthin. Quantum-yields dependence of solvent, temperature, and wavelength of irradiation in relation to packaging and storage of carotenoid pigmented salmonoids. Z. Lebensm. Unters. Forsch. 192: 433–439.

Christophersen, A.G., Bertelsen, G., Andersen, H.J., Knuthsen, P., and Skibsted, L.H. 1992. Storage life of frozen salmonoids. Effect of light and packaging conditions on carotenoid oxidation and lipid oxidation. Z. Lebensm. Unters. Forsch. 194: 115–119.

Cronin, D.A., Powell, R., and Gormley, R. 1991. An examination of the n-3 and n-6 polyunsaturated fatty acid status of wild and farmed Atlantic salmon (*Salmo salar*). Irish J. Food Sci. Technol. 15: 53–62.

Erickson, M.C. 1993. Compositional parameters and their relationship to oxidative stability of channel catfish. J. Agric. Food Chem. 41: 1213–1218.

Fakourelis, N., Lee, E.C., and Min, D.B. 1987. Effects of chlorophyll and β-carotene on the oxidation stability of olive oil. J. Food Sci. 52: 234–235.

Frankel, E.N. 1991. Recent advances in lipid oxidation. J. Sci. Food Agric. 54: 495–511.
Frigg, M., Prabucki, A.L., and Ruhdel, E.U. 1990. Effect of dietary vitamin E levels on oxidative stability of trout fillets. Aquaculture 84: 145–158.
Gillbro, T. and Cogdell, R.J. 1989. Carotenoid fluorescence. Chem. Phys. Lett. 158: 312–316.
Gloria, M.B.A., Grulke, E.A., and Gray, J.I. 1993. Effect of type of oxidation on beta-carotene loss and volatile products formation in model systems. Food Chem. 46: 401–406.
Gormley, T.R. 1992. A note on consumer preference of smoked salmon colour. Irish J. Agric. Food Res. 31: 199–202.
Haard, N.F. 1992. Control of chemical composition and food quality attributes of cultured fish. Food Res. Int. 25: 289–307.
Halevy, O. and Sklan, D. 1987. Inhibition of arachidonic acid oxidation by β-carotene, retinol and α-tocopherol. Biochim. Biophys. Acta 918: 303–307.
Hirayama, O., Nakamura, K., Hamada, S., and Kobayasi, Y. 1994. Singlet oxygen quenching ability of naturally occurring carotenoids. Lipids 29: 149–150.
Hwang, K.T. and Regenstein, J.M. 1988. Protection of menhaden lipids from rancidity during frozen storage. J. Food Sci. 54: 1120–1124.
Iliou, J.-P., Jourd'heuil, D., Robin, F., Serkitz, B., Guivarc'h, P., Volland, J.-P., and Vilaine, J.-P. 1992. Kinetics of photoperoxidation of arachidonic acid: Molecular mechanism and effects of antioxidants. Lipids 27: 959–967.
Ingemansson, T., Pettersson, A., and Kaufmann, P. 1993. Lipid hydrolysis and oxidation related to astaxanthin content in light and dark muscle of frozen stored rainbow trout (*Oncorhynchus mykiss*). J. Food Sci. 58: 513–518.
Jensen, N.-H., Nielsen, A.B., and Wilbrandt, R. 1982. Chlorophyll α sensitized *trans-cis* photoisomerization of all-*trans*-β-carotene. J. Am. Chem. Soc. 104: 6117–6119.
Jonsson, L. 1991. Thermal degradation of carotenes and influence on their physiological functions. In *Nutritional and Toxicological Consequences of Food Processing*, M. Friedman (Ed.), p. 75–82, Plenum Press, New York.
Jørgensen, K. and Skibsted, L.H. 1990. Light sensitivity of carotenoids used as food colours. Quantum-yields dependence of wavelength and oxygen pressure for direct and sensitized photodegradation of solubilized lutein and β-carotene. Z. Lebensm. Unters. Forsch. 190: 306–313.
Jørgensen, K. and Skibsted, L.H. 1993. Carotenoid scavenging of radicals. Effect of carotenoid structure and oxygen partial pressure on antioxidative activity. Z. Lebensm. Unters. Forsch. 196: 423–429.
Josephson, D.B., Lindsay, R.C., and Stuiber, D.A. 1991. Volatile carotenoid-related oxidation compounds contributing to cooked salmon flavor. Lebensm. Wiss. Technol. 24: 424–432.
Jung, M.Y. and Min, D.B. 1991. Effects of quenching mechanisms of carotenoids on the photosensitized oxidation of soybean oil. J. Am. Oil Chem. Soc. 68: 653–658.
Keller, H.E. 1988. *Analytical Methods for Vitamins and Carotenoids in Feed.* Hoffmann-La Roche, Basel, Switzerland, Index no. 2101.
Kornhauser, A., Wamer, W., and Lambert, L. 1989. β-Carotene protection against phototoxicity *in vivo*, in *Carotenoids. Chemistry and Biology*, N.I. Krinsky, M.M. Mathews-Roth, and R.F. Taylor (Ed.), p. 301–312. Plenum Press, New York.
Krinsky, N.I. 1989. Antioxidant functions of carotenoids. Free Radical Biol. Med. 7: 617–635.
Kubow, S. 1993. Lipid oxidation products in food and atherogenesis. Nutr. Rev. 51: 33–40.
Lewis, B.L., Harrison, D.L., and Sanford, P.E. 1958. Effect of xanthophyll on the palatability and fat stability of fresh and frozen broad-breasted bronze turkeys. Food Res. 23: 7–16.
Love, R.M. 1988. *The Food Fishes, Their Intrinsic Variation and Practical Implications.* Farrand Press, London.
McCallum, I.M., Cheng, K., and March, B.E. 1987. Carotenoid pigmentation in two strains of chinook salmon (*Oncorhynchus tschawytscha*) and their crosses. Aquaculture 67: 291–300.
Miki, W. 1991. Biological functions and activities of animal carotenoids. Pure Appl. Chem. 63: 141–146.
Murasecco-Suardi, P., Oliveros, E., Braun, A.M., and Hansen, H.-J. 1988. Singlet-oxygen quenching by carotenoids: Steady-state luminescence experiments. Helv. Chim. Acta 71: 1005–1010.
Najar, S.V., Bobbio, F.O., and Bobbio, P.A. 1988. Effects of light, air, antioxidants and pro-oxidants on Anatto extracts (*Bixa orellana*). Food Chem. 29: 283–289.
No, H.K. and Storebakken, T. 1991. Color stability of rainbow trout fillets during frozen storage. J. Food Sci. 56: 969–972, 984.
Obach, A. and Baudin Laurencin, F. 1992. Effects of dietary oxidized fish oil and deficiency of antioxidants on the immune response of turbot, *Scophthalmus maximus*. Aquaculture 107: 221–228.
Ostrander, J., Martinsen, C., Liston, J., and McCullough, J. 1976. Sensory testing of pen-reared salmon and trout. J. Food Sci. 41: 386–390.
Pesek, C.A. and Warthesen, J.J. 1987. Photodegradation of carotenoids in a vegetable juice system. J. Food. Chem. 52: 744–746.
Pesek, C.A. and Warthesen, J.J. 1990. Kinetic model for photoisomerization and concomitant photodegradation of β-carotenes. J. Agric. Food Chem. 38: 1313–1315.
Pesek, C.A., Warthesen, J.J., and Taoukis, P.S. 1990. A kinetic model for equilibration of isomeric β-carotenes. J. Agric. Food Chem. 38: 41–45.
Polvi, S.M. and Ackman, R.G. 1992. Atlantic salmon (*Salmo salar*) muscle lipids and their response to alternative dietary fatty acid sources. J. Agric. Food Chem. 40: 1001–1007.
Polvi, S.M., Ackman, R.G., Lall, S.P., and Saunders, A.L. 1991. Stability of lipids and omega-3-fatty acids during frozen storage of Atlantic salmon. J. Food Processing 15: 167–181.
Pozo, R., Lavety, J., and Love, R.M. 1988. The role of dietary α-tocopherol (Vitamin E) in stabilizing the canthaxanthin and lipids of rainbow trout muscle. Aquaculture 73: 165–175.
Röpke, A. 1988. Canthaxanthin concentration in different coloured fillets of farmed rainbow trout (*Salmo gairdneri* R.) in relation to dietary doses. Int. Council Explor. Sea, Maricult. Committ., F:34, 7pp.
Sänger, A.M. 1993. Limits to the acclimation of fish muscle. Rev. Fish Biol. Fish. 3: 1–15.
SAS Institute Inc. 1990. *SAS/STAT User's Guide. Version 6, 4th ed.* SAS Institute Inc., Cary, NC.
Sattar, A., DeMan, J.M., and Alexander, J.C. 1977. Wavelength effect on light-induced decomposition of vitamin A and β-carotene in solutions and milk fat. J. Inst. Can. Sci. Technol. Aliment. 10: 56–60.
Sigurgisladottir, S., Parrish, C.C., Lall, S.P., and Ackman, R.G. 1994. Effects of feeding natural tocopherols and astaxanthin on Atlantic salmon (*Salmo salar*) fillet quality. Food Res. Int. 27: 23–32.
Storebakken, T. and No, H.K. 1992. Pigmentation of rainbow trout. Aquaculture 100: 209–229.
Terao, J. 1989. Antioxidant activity of β-carotene-related carotenoids in solution. Lipids 24: 659–661.
Torrissen, O.J., Hardy, R.W., and Shearer, K.D. 1989. Pigmentation of salmonids—Carotenoid deposition and metabolism. CRC Crit. Rev. Aquat. Sci. 1: 209–225.
Truscott, T.G. 1990. The photophysics and photochemistry of the carotenoids. J. Photochem. Photobiol. B: Biol. 6: 359–371.
Vyncke, W. 1970. Direct determination of the thiobarbituric acid value in trichloroacetic acid extracts of fish as a measure of oxidative rancidity. Fette Seifen Anstrichm. 72: 1084–1087.
Waagbø, R., Sandnes, K., Torrissen, O.J., Sandvin, A., and Lie, Ø. 1993. Chemical and sensory evaluation of fillets from Atlantic salmon (*Salmo salar*) fed three levels of *n*-3 polyunsaturated fatty acids at two levels of vitamin E. Food Chem. 46: 361–366.
Wasielewski, M.R., Johnson, D.G., Bradford, E.G., and Kispert, L.D. 1989. Temperature dependence of the lowest excited singlet-state lifetime of all-*trans*-β-carotene and fully deuterated all-*trans*-β-carotene. J. Chem. Phys. 91: 6691–6697.
Will, O.H. and Scovel, C.A. 1989. Photoprotective functions of carotenoids, in *Carotenoids. Chemistry and Biology*, N.I. Krinsky, M.M. Mathews-Roth, and R.F. Taylor (Ed.), p. 229–236, Plenum Press, New York.
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Cooked Blue Crab Claw Meat Aroma Compared with Lump Meat

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ABSTRACT

Sensory evaluation of claw and lump crabmeats by sniffing indicated no aroma difference ($p > 0.05$); however, differences ($p < 0.05$) were found between the taste and aromatics of aqueous extracts of both meats. Eleven aroma notes (combined total) were found in the meats by aroma extract dilution analysis (AEDA). Flavor dilution factors for compounds common to both meats were not statistically different ($p > 0.05$). Aroma notes found to have highest intensity in both meats were: meaty/salty/soy sauce [3-(methylthio)-propanal], sweet/fruity, metallic/rubber/gasoline, nutty/popcorn [2-acetyl-1-pyrroline], nutty/popcorn, pine/sweet/hay/dry seaweed, and mushroom/solvent.

Key Words: blue crab, lump meat, claw meat, crab aroma

INTRODUCTION

MANY CRAB PROCESSORS cook live crabs the day before meat-picking allowing them to cool overnight. After picking, crabmeat is generally divided according to the anatomical region from which it originates, e.g., claw, body (lump) and white meats. Crab connoisseurs claim that the flavor of meat from various parts of the crab is different, yet little scientific evidence supports this claim.

Volatile components in cooked crabmeat have been studied by several investigators (Rayner et al., 1981; Hsieh et al., 1989; Flament, 1990; Matiella and Hsieh, 1990; Chung and Cadwallader, 1993). Hayashi et al. (1981) prepared a satisfactory synthetic extract of boiled snow crab meat from 12 synthetic chemicals based on results from triangle difference tests (Jellinek, 1985).

Aroma extract dilution analysis (AEDA) has been used widely in screening important aromas in an extract (Schieberle and Grosch, 1987; Gasser and Grosch, 1988; Blank et al., 1992). The aroma intensity unit is usually expressed as the flavor dilution (FD)-factor. This is defined as the ratio of the concentration of a compound in the initial extract to that in the most diluted extract in which the odor was detected by gas chromatography/olfactometry (GC/O) (Blank and Grosch, 1991). Our objective was to determine whether the flavor of lump and claw meats of blue crab differ as evaluated by sensory evaluation and AEDA.

MATERIALS & METHODS

Materials

Cooked blue crab claw and lump meats were purchased three separate times between August and September 1993 from a local seafood retail outlet in Baton Rouge, La. Meats were transported on ice to the LSU Dept. of Food Science and stored at 4°C until analyzed (less than 5 hr). All standard flavor compounds were purchased from Aldrich Chemical Co. (Milwaukee, WI), except 2-acetyl-1-pyrroline (2-AP) obtained from Dr. R. Buttery (USDA, ARS, WRRC, New Albany, CA).

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Sensory evaluation of crab meats

To determine possible flavor differences between crab claw and lump meats, two sensory evaluation experiments were carried out. Preliminary experiments suggested that warm crabmeat was preferred by the sensory panel over refrigerated or cold crabmeat when aroma quality was evaluated. Subsequent experiments utilized warm (≈75°C) samples. Furthermore, due to the distinct texture and color differences between the two crabmeats, panelists could distinguish samples even under red light or blindfolded. Therefore, experiments were carried out such that the effects of both qualities were eliminated or minimized. In this, aqueous crabmeat extracts were prepared to eliminate texture differences, and sample tubes were covered with aluminum foil to mask color differences. The sensory panel consisted of seven to 10 volunteers of faculty and students within the LSU Dept. of Food Science. All panelists had prior sensory evaluation experience. Evaluations were conducted on meats purchased from the same seafood outlet on the same day.

Meat evaluation

Each portion (≈5g) of claw or lump meat was individually wrapped in aluminum foil, vacuum-packaged in a plastic bag (≈5 cm × 7 cm) and randomly numbered. All samples were cooled on ice during preparation and were evaluated within 24 hrs. During each session, four triangle difference tests, consisting of two sets of duplicate samples, were performed by panelists. Samples were warmed in a water bath (≈75°C) for 10–15 min before random distribution to panelists. Evaluation was performed in a temperature-regulated (23°C) room under red light to mask possible bias from package or sample color. Panelists were asked to massage each sample with their fingers to simulate chewing before cutting open the package and evaluating its aroma.

Extract evaluation

Samples of each crabmeat (300g) plus 500 mL water were homogenized using a mixer (Cat. no. DIM24, General Electric, Bridgeport, CT) at low speed for 10 min in a stainless steel mixing bowl. Aqueous extract was obtained by filtering the homogenate through three layers of cheese cloth. Extract was further clarified by centrifugation at 3500 rpm (2000 × *g*) for 15 min using a RC5C Sorvall centrifuge (Ivan Sorvall, Inc., Norwalk, CT). Aqueous extract (200 mL) was transferred to a ≈240 mL glass jar and tightly capped. Contents were then pasteurized ($T_{initial}$ = 21°C, T_{final} = 96°C) by immersing the jar (water level above contents) for 15 min in a boiling waterbath (100°C) before transferring to culture tubes (7 mL/tube). Each tube was sealed with plastic wrap and the bottom half covered with aluminum foil to minimize bias from any color differences. Testing conditions were previously described. Panelists were instructed to place the entire contents of each tube into the mouth and evaluate taste. In order to eliminate the perception of the aromatics, panelists were instructed to pinch their nose and inhale and exhale using the mouth only. Water was provided to panelists for mouth rinsing after each evaluation.

Additional experiments were carried out to determine whether such aqueous extracts affected perception of aromatics by instructing the panel to place the sample in the mouth, hold it for 3 s, and exhale slowly through the nose (Jellinek, 1985). Data from all sensory evaluations were analyzed statistically (Roessler et al., 1978).

Extraction of water-soluble flavor components

A sample of 200g crabmeat was ground manually in a 2-L glass beaker using a pestle. Distilled water (200 mL) was added to facilitate extraction of water-soluble flavor components. The aqueous extract was recovered by pressing and filtering the mixture through three layers of cheese cloth into a 200-mL volumetric flask. The extract was further

Table 1—Results of triangle difference tests on blue crab claw and lump meats and their respective aqueous extracts

Type of Evaluation	Sample form	No. of panelists	No. of tests[a]	No. of Judgements		Level tested[b]
				Total	Correct	
Aroma						
trial 1	meat	8	4	32	10	0.05 (−)[c]
trial 2	meat	9	4	36	16	0.05 (−)
Taste						
trial 1	extract	7	4	28	15	0.05 (+)
trial 2	extract	10	4	40	30	0.001 (+)
Aromatics						
trial 1	extract	7	4	28	15	0.05 (+)
trial 2	extract	10	4	40	22	0.05 (+)

[a] Number of tests per panelist
[b] Level of probability tested. Data were analyzed using table of significance in triangle testing of Roessler et al. (1978)
[c] (−) = not significantly different, (+) = significantly different

clarified by centrifugation at 3500 rpm (2000 × *g*) for 10 min and with 10 mL hexane to remove any oily pigment. Liquid supernatant was transferred into a 500-mL separatory funnel and extracted with a total of 200 mL (10 × 20 mL) of 10% methanol in dichloromethane. The volume of solvent extract was reduced to 5 mL under a gentle stream of nitrogen, dried over 12-g anhydrous sodium sulfate, and further reduced to 0.4 mL.

Gas chromatography/mass spectrometry and flame ionization detection/olfactometry (GC/MS-FID/O)

The GC/MS system consisted of an HP 5790A GC equipped with flame ionization detector (FID) and an HP 5970B mass selective detector (MSD) (Hewlett-Packard Co., Palo Alto, CA). Five µL of each extract was injected (splitless mode; 200°C injector; 1 min valve delay) simultaneously into dual (closely matched) fused silica open tubular (FSOT) columns (Supelcowax 10, 60 m length × 0.25 mm i.d. × 0.25 µm film thickness; Supelco, Inc., Bellefonte, PA). Injector effluent, after passing through a FSOT precolumn (1m length × 0.25 mm i.d. × 0.25 µm film thickness), was split 1:1 to each column using a glass Y-splitter. Column A was connected to the MSD, while the end of column B was split 1:1 to an FID and sniffing port supplied with humidified air. FID was 250°C and sniffing port transfer line was 200°C. Helium was carrier gas at a linear velocity of 25 cm/s. It was necessary to partially restrict the flow between column A and MSD (using ≈1m length × 0.1 mm i.d. FSOT column) in order to achieve the same linear velocity for both columns. Oven temperature was programmed from 40 to 195°C at 2.0°C/min with initial hold of 5 min and final hold of 40 min. MSD conditions were as follows: capillary direct MS interface temperature, 200°C; ion source temperature, 200°C; ionization voltage, 70 eV; mass range, 33–290 a.m.u.; scan rate, 1.67 scans/sec; and electron multiplier voltage, 1800 V.

Aroma extract dilutions

Serial dilutions were made from the 0.4 mL concentrated extracts in the ratio of 1:1 using dichloromethane as diluent. Each dilution was transferred to a new 2-mL amber vial equipped with a teflon-lined screw cap. Diluted solutions were stored at −80°C until analyzed.

Gas chromatography/olfactometry (GC/O)

The GC/O system consisted of an HP 5790 GC (Hewlett-Packard Co., Palo Alto, CA) equipped with a FSOT column (Supelcowax 10; 60 m × 0.32 mm i.d. × 0.25 µm film thickness; Supelco, Inc., Bellefonte, PA), an FID, and a sniffing port. GC conditions were as described except that oven temperature was programmed from 40°C to 195°C at 5°C/min with initial hold 5 min and final hold 40 min. GC/O was performed by two panelists familiar with crab flavor and the olfactometry technique. Each sample was replicated three times.

The average FD-factor of each flavor note was calculated by the arithmetic mean (N) of the $\log_2$ (FD-factor). (N) was readily converted back to FD-factor by: FD-factor = 2^N. Means were analyzed for statistical differences by a two-tailed t-test using SigmaPlot™ software (v. 4.0; Jandel Scientific, Corte Madera, CA.). Differences were considered significant when means of compared sets differed at $p < 0.05$.

Compound identification

Identifications were based on comparison of GC retention indices (RI) (van den Dool and Kratz, 1963), mass spectra, and aroma properties of unknowns with those of authentic standard compounds analyzed under identical experimental conditions

RESULTS & DISCUSSION

Aroma and taste of claw and lump crabmeats

Sensory evaluation results (Table 1) indicated no differences ($p > 0.05$) between aromas of claw and lump crabmeats (meat experiment). However, when aqueous extracts of each meat were presented to panelists for tasting while blocking their nose most could detect the odd sample, i.e., the tastes were different ($p < 0.05$). This suggests that aroma alone was not sufficient to differentiate between the two meats. However, it is not clear whether the difference detected in the extracts was due to taste sensation alone, since evaluation of aromatics of aqueous extracts of the two meats showed the extracts were different ($p < 0.05$).

Farragut (1964) determined the proximate composition of blue crab in the Chesapeake Bay over a 1-yr period. During the

Table 2—Odorants detected in extracts of blue crab claw and lump meats by aroma extract dilution analysis

No.	Aroma description	RI/RT[a]	Average Log_2 (FD-factor)[b]				Compound
			Claw	%CV[c]	Lump	%CV	
1	fishy/ammonia	(4.8 min)	—[d]	—	0.50	115	trimethylamine
2	mushroom/solvent	(7.4 min)	3.00	62	2.50	83	unknown
3	creamy/sour	(9.4 min)	—	—	0.50	115	2,3-butanedione
4	alkaline/rancid meal/yolk	1008	0.67	87	1.67	124	pyrrolidine
5	nutty/popcorn	1336	3.67	37	3.33	77	2-acetyl-1-pyrroline
6	metallic/rubber/gasoline	1372	3.67	75	4.33	24	unknown
7	rice/popcorn	1414	—	—	1.50	115	unknown
8	meaty/salty/soy sauce	1455	5.00	57	7.33	7	3-(methylthio)-propanal
9	pine/sweet/hay/dry seaweed	1724	2.80	30	3.17	42	unknown
10	nutty/popcorn	1766	3.00	52	3.33	90	unknown
11	sweet/fruity	2046	4.17	28	4.83	36	unknown

[a] RI = retention index; RT = retention time. Numbers in parentheses represent average retention times.
[b] Averages for claw and lump meats were not statistically different ($p > 0.05$) by t-test.
[c] %CV = percent coefficient of variation (n = 6).
[d] Odorant below detection limit.

months of August and September, the body meat had 15.6% protein, 81.65% moisture, 1.05% oil and 1.57% ash on a wet-weight basis, while the claw meat had 15.5%, 82.4%, 1.0%, and 1.68%, respectively. The proximate compositions of the two types of meat were not notably different. However, regarding specific chemical components, differences between lump and claw meats may be considerable. George and Gopakumar (1987) compared the biochemical composition of claw and body meats of sand crab (*Scylla serrata*) and detected major differences in concentrations of glycogen, phosphorus, pentose, salt, protein, and amino acids. Body meat contained higher concentrations of glycogen, phosphorus, potassium, sarcoplasmic protein, and glycine, alanine, and aspartic acid. Claw meat contained more pentose, sodium, and amino acids such as valine, methionine, etc. Slight differences in composition would be expected for the two types of blue crabmeats. In addition to the noticeable taste difference, both meats had distinct texture and color differences. Wasserman and Talley (1968) discussed the influence of texture, color, and preparation of meat on olfactory recognition. Therefore, we took precautions to minimize or obscure the texture and color differences during sensory evaluation.

Additional experiments with panelists holding the extract a short time in the mouth and exhaling headspace from the nose (extract experiment) had surprising results. Panelists could distinguish differences ($P < 0.05$) based on the aromatics of the two extracts. Apparently, this contradicted results obtained in the meat experiment. During breathing, inhaled air generally bypasses the regio olfactoria at the top inside of the nose where odor is sensed (Jellinek, 1985). When extracts of both meats were used, the odorant might be more readily released from the matrix in the mouth and create a more concentrated headspace of odorants. This, coupled with the exhaling action, enabled the concentrated mass of odorants, to pass the regio olfactoria for a more intense sensation. Furthermore, it is possible that redistribution or differential adsorption of volatiles occurred in the mouth and nasal passageway resulting in aromatics of the two samples being perceived as different (Moncrieff, 1955; Mozell, 1970).

Results of aroma evaluation were confirmed by AEDA results. The Log_2 (FD-factor) profiles of the two types of crabmeat were similar (Table 2). Eight characteristic aroma notes were detected in claw and 11 in the lump crabmeats. A salty/meaty/soy sauce-like note (No. 8) was the most intense aroma in both meats followed by a sweet/fruity note (No. 11), a metallic/rubber/gasoline-like note (No. 6), and two nutty/popcorn-like notes (No. 5 and 10). In general, intensities of aroma notes common to both meats were higher in the lump crabmeat. The major difference in aroma sensation might be largely due to the 3 odorants in lump meat with fishy/ammonia-like (No. 1), creamy/sour (No. 3), and rice/popcorn-like (No. 7) notes. These low-intensity notes might also be the cause of insensitivity by panelists in the meat experiment but not in the latter part of the extract experiment.

Odorous compounds from aroma extract dilution analysis (AEDA)

Whitfield et al. (1988) detected an iodoform-like off-flavor in some Australian crustacea and identified 2,6-dibromophenol as the source. This compound also was reported in marine algae and bryozoa on which the crustacea feed. Boyle et al. (1992) identified a group of bromophenols in saltwater Pacific salmon as well as other seafoods. However, they reported that the sea-, brine-, iodine-like flavor notes were desirable and associated with saltwater fish and seafoods. They further reported detecting such compounds in high concentrations in blue crab. However, in our experiment, those compounds were not detected.

The compound with the highest average Log_2 (FD-factor) value was described as having a salty, meaty, soy sauce-like note (no. 8) in both types of crabmeat and was identified as 3-(methylthio)-propanal (Table 2). This compound had a very low odor threshold (0.2 ppb, Guadagni et al., 1972) and may be formed via Strecker degradation from methionine (Morton et al., 1960).

Another interesting note was described as having a sweet/fruity (no. 11) aroma and eluted very late during GC/O. This note, along with no. 5 and 6, had high FD-factors. Note no. 6 had a metallic/rubber/gasoline-like aroma. Under our chromatographic conditions, this note had an undesirable aroma. Nevertheless, it could be diminished or masked by the other, more desirable notes such as notes no. 8 and 11. Metallic and fishy odorants have been reported in oxidized butterfat by some researchers (Peers and Swoboda, 1977; Swoboda and Peers, 1977). Their investigations with a model system led them to conclude that both 1-octen-3-one and 1-octa-*cis*-5-dien-3-one were responsible for metallic odor. In high concentrations, only 1-octen-3-one exhibits a metallic aroma; however, 1-octa-*cis*-5-dien-3-one produces this aroma at concentrations as low as 1 to 10^{12} dilutions. Since our study involved cooked crabmeat, thermally generated compounds were expected to contribute to crab aroma. The compound 2-acetyl-1-pyrroline (no. 5) was identified as imparting a nutty/popcorn-like note to the sample. Buttery et al. (1983) identified this compound in aromatic rice, and its odor threshold was reported to be 0.1 ppb.

We were uncertain why trimethylamine (TMA) was detected only in lump meat and its intensity was among the lowest. Josephson and Lindsay (1986) reported that TMA was an important contributor to overall boiled crab-type aromas of fresh crab. Chung and Cadwallader (1993) compared the volatile compounds of blue claw meat and its processing byproduct in which the average concentrations of TMA were 230 and 174 ng/g, respectively.

Pyrrolidine (no. 4) was among the weakest aroma notes detected in both meats. This compound could have been thermally produced through Strecker degradation of proline or by bacteria (Allison and MacFarlane, 1989; Griffith and Hammond, 1989). Another thermally generated compound was 2,3-butanedione (no. 3), which was described as creamy and sour. Like TMA, this compound was identified only from the lump meat.

CONCLUSIONS

DIFFERENCES in the flavor of claw and lump crabmeat were confirmed. This could be attributed to both volatile components and taste-active components. This was supported by the dissimilarity detected in the number of odorants found in the two crabmeat samples by AEDA. However, Log_2 (FD-factors) for aroma notes common to both meats were similar.

REFERENCES

Allison, C. and MacFarlane, G.T. 1989. Influence of pH, nutrient availability, and growth rate on amine production by *Bacteroides fragilis* and *Clostridium perfringens*. Appl. Environ. Microbiol. 55: 2894–2898.

Blank, I. and Grosch, W. 1991. Evaluation of potent odorants in dill seed and dill herb (*Anethum Graveolens* L.) by aroma extract dilution analysis. J. Food Sci. 56: 63–67.

Blank, I., Sen, A., and Grosch, W. 1992. Potent odorants of the roasted powder and brew of Arabica coffee. Z. Lebesm. Unters. Forsch. 195: 239–245.

Boyle, J.L., Lindsay, R.C., and Stuiber, D.A. 1992. Bromophenol distribution in salmon and selected seafoods of fresh- and saltwater origin. J. Food Sci. 57: 918–922.

Buttery, R.G., Ling, L.C., Juliano, B.O., and Turnbaugh, J.G. 1983. Cooked rice aroma and 2-acetyl-1-pyrroline. J. Agric. Food Chem. 31: 823-826.

Chung, H.Y. and Cadwallader, K.C. 1993. Volatile components in blue crab (*Callinectes sapidus*) meat and processing by-product. J. Food Sci. 58: 1203–1207, 1211.

Farragut, R.N. 1964. Proximate composition of Chesapeake Bay blue crab (*Callinetes sapidus*). J. Food Sci. 30: 538–544.

Flament, I. 1990. Analysis of boiled crab meat flavour: identification of new alcoholic constituents. Lebensm.-Wiss. u.-Technol. 23: 274–275.

Gasser, U. and Grosch, W. 1988. Identification of volatile flavour compounds with high aroma values from cooked beef. Z. Lebensm. Unters. Forsch. 186: 489–494.

George, C. and Gopakumar, K. 1987. Biochemical studies on crab *Scylla Serrata*. Fishery Technol. 24: 57–61.

Griffith, R. and Hammond, E.G. 1989. Generation of Swiss cheese flavor components by the reaction of amino acids with carbonyl compounds. J. Dairy Sci. 72: 664–613.

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Surimi-Based Imitation Crab Characteristics Affected by Heating Method and End Point Temperature

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ABSTRACT

Sensory panel scores and Instron compression tests indicated baked and microwaved imitation crab legs were softer ($P < 0.05$) than unheated controls. Sensory scores further indicated that baked samples were softer ($P < 0.05$) and less chewy than microwaved samples. End point temperature to which samples were heated (71°C and 93°C) had no effect on results. No differences in moisture content were found among samples, but heated samples were lower ($P < 0.05$) in expressible moisture than unheated controls. Heating may have caused free water to rebind with starches in the product, thus decreasing expressible moisture, which contributed to decreases in firmness and chewiness.

Key Words: imitation crab, texture, flavor, heating effects

INTRODUCTION

SURIMI, deboned fish which has been washed repeatedly and mixed with cryoprotectants to prevent protein denaturation during frozen storage (Lee, 1984; Lanier, 1986), has excellent texturizing, gelling, and binding properties (Lee, 1984; Lanier, 1986). Its high concentration of myofibrillar protein enables the product to gel upon heating to produce a chewy, elastic texture (Lee, 1986; Lanier, 1986; Shimizu, 1985). This characteristic makes surimi ideal for fabricating new food items which require specific textural attributes, such as simulated shellfish. The most popular such product from surimi is imitation crab meat (Vondruska, 1985; Anonymous, 1987).

Throughout production, processing temperatures are important in development of gel structure and resulting textural characteristics of imitation crab. Finished product is strongly influenced by cooking temperature and length of time heat is applied during processing (Lee, 1984; Wu et al., 1985a, b, c; Douglas-Schwarz and Lee, 1988; Montejano et al., 1984). Excessive heating produces a tough, rubbery analog (Lee, 1986) which becomes increasingly firm, opaque, and brittle as heating continues (Lanier et al., 1982). Inadequate cooking results in an undesirable soft texture (Lee, 1984). However, little is known about effects additional heat processing in the home may have on physical properties of products such as imitation crab.

Although imitation crab is designed to be served cold, consumers may substitute it in recipes which require further heating. Although much research has been reported on the effects of heat on surimi during production of the analog, data are lacking on effects of further heating on physical characteristics of imitation crab analogs. Our objective was to determine the effects of baking and microwave heating to end point temperatures of 71°C and 93°C on physical and sensory characteristics of imitation crab compared to unheated controls.

MATERIALS & METHODS

Production and sample preparation of imitation crab

Grade A Alaskan pollack (*Theragra chalcogramma*) surimi (Great Land Seafoods, Kodiak, AK), containing 8% sorbitol and 0.3% phosphate as a 50/50 blend of sodium tripolyphosphate and tetrasodium pyrophosphate, was used to produce leg style imitation crab. The sample was commercially produced (UniSea Foods, Inc., Redmond, WA) using standard production techniques and a formula developed at the Univ. of Maryland (College Park, MD). In addition to surimi (57.2%), ingredients included potato starch (1.05%, Nonpariel Corp., Blackfoot, ID), wheat starch (1.05%, Aytex P, Ogilvie Mills, Inc., Minnetonka, MN), modified cornstarch (3.16%, Purity W, hydroxypropyl di-starch phosphate, National Starch and Chemical Corp., Bridgewater, NJ), liquid egg white (10.0%), salt (1.32%, Morton International, Chicago, IL), natural and artificial crab flavors (1.0%, T. Hasegawa, Cerritos, CA), chilled water (25.2%), monosodium glutamate (0.028%, Ajinomoto Co., Inc., Teaneck, NJ), and inosine 5'-monophosphate (0.002%, Ajinomoto Co., Inc., Teaneck, NJ) based on total formula weight. The product was frozen at −24°C immediately after production and transported at −20 ± 1°C by surface transportation to the University of Maryland, where it was immediately placed in frozen storage and maintained at −20 ± 2°C until further heat treatment.

Table 1—Procedures for heating imitation crab samples to end-point temperatures of 71°C and 93°C by baking and microwave heating

Heating Method	Directions
Unheated control	Product allowed to equilibrate to room temperature prior to testing.
Baked to 71°C	21 ½ min at 177°C; Rotate dish 180° at 11 min; Cool to room temperature.
Baked to 93°C	34 min at 177°C; no rotation; Cool to room temperature.
Microwaved to 71°C	4 min @ 70% power; 3 min standing time; 4 min @ 70% power; 3 min standing time; Cool to room temperature.
Microwaved to 93°C	6 min @ 70% power; 2 min standing time; 5¾ min @ 70% power; 2 min standing time; Cool to room temperature.

Imitation crab legs were chosen randomly from frozen storage, defrosted overnight at refrigeration temperature (2 ± 2°C), cut in half, and divided into five treatment groups. They were: baked to 71°C (Baked 71), baked to 93°C (Baked 93), microwaved to 71°C (Microwaved 71), microwaved to 93°C (Microwaved 93), and an unheated control. End point temperatures were chosen to simulate in-home heat processing conditions. Each half-leg sample weighed ≈18 g and was about 2.0 cm in diameter. Some variations in leg diameters were observed after heating. Surface areas of baked samples showed a mean variation of 2.1% whereas microwaved samples varied by 2.5%. Unheated controls were kept refrigerated while treated samples were heated then equilibrated to room temperature (21°C ± 2°C) prior to testing.

Baked samples were prepared by arranging 34 half-legs of imitation crab on a wire rack set into a 23 cm × 33 cm × 5 cm Pyrex (Corning Glass Works, Corning, NY) baking dish to which 250 mL boiling water was added. Microwaved samples were prepared by placing 34 half-legs of imitation crab in 25 cm × 25 cm × 5 cm baking dishes (Corning Glass Works). Each sample dish was covered with foil (baked samples) or plastic wrap (microwaved samples) and punctured near each end to allow steam to escape during heating. Baked samples were heated in a conventional oven (Model KC535ADH, White Westinghouse Corporation, Pittsburgh, PA) preheated to 177°C. Microwaved samples were heated on the turntable of a conventional microwave oven (Sharp, Model R-5A52; Sharp Electronic Corp., Mahwah, NJ). After heating, all samples were cooled to room temperature (21°C ± 2°C) prior to testing.

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End point temperatures of 71°C or 93°C (±3°C) were achieved using methods established during pretesting (Table 1) with a Fluke digital thermometer and recorder (scanner Model 2300A, digital thermometer Mocel 2190A; John Fluke Manufacturing Co. Inc., Everett, WA). Six thermocouples were calibrated prior to testing using ice and boiling water. For baked samples, thermocouples were randomly placed through the top of selected legs and remained in those positions for continuous temperature monitoring throughout heating. For microwaved treatments, thermocouples were randomly placed in selected legs immediately after heating to monitor final temperatures. Final end point temperatures were recorded upon standing 2–3 min after removal from the microwave (Table 1). Repeated testing of the methods (Table 1) consistently resulted in temperatures of 71°C and 93°C (±3°C).

Physical analysis

Center sections of heated and unheated samples equilibrated to room temperature were cut into 2-cm lengths using a custom made cutting box and placed in an upright position on the platform of an Instron Universal Testing Machine (Model A1026C; Instron Corp., Canton, MA). Compression to 90% deformation was achieved using a 50 kg load cell and a 3.5 cm diameter compression test cell. Crosshead and chart speeds were maintained at 5 cm/min, and one complete downward and upward cycle of the Instron was performed on each sample. Two replications of five samples from each treatment were conducted. Fracturability (kg) and harcness (kg) were measured following the methods of Bourne (1978). Fracturability of imitation crab was defined as the force measured at the first peak of the Instron curve (Fig. 1). The force at this point is commonly used to measure fracturability.

Expressible moisture was determined using Instron conditions described. Two-cm samples were compressed to 90% deformation on previously dried and weighed Whatman #1 filter paper. Wet filter paper was reweighed, and moisture expressed was reported as a percent moisture content of the sample. Two replications of five samples from each treatment were tested.

Hunter values were determined on both interior and exterior surfaces of samples using a Pacific Scientific colorimeter (Gardner Neotec, Silver Spring, MD) equipped with a Spectrogard Color System and Spectrogard Color System Version 1.1 software. Illuminant C with a 10° standard observer was utilized, and the instrument was calibrated with black and white calibration standards supplied with the instrument. Exterior measures were made by manually pressing two samples side-by-side against the 22 mm diameter light aperture. For interior surface measures, leg samples were unrolled, folded four layers thick, and manually pressed against the light aperture. Two replications of three samples from each treatment were performed. Using Hunter L, a, and b values, whiteness index was calculated using the method which Gates and Parker (1992) used to evaluate whiteness of blue crab.

Chemical analysis

Two replications of duplicate samples from each treatment were analyzed for lipid, protein, and ash contents. Total lipid content was determined by the method of Bligh and Dyer (1959). Lipids were extracted using a chloroform/methanol solvent system. Extracts were dried at 45°C using a vacuum evaporator (Rotovapor Model RE-111; Buchi, Flawil, Switzerland). Protein was analyzed using the Kjeldahl method (AOAC, 1980). Quantification of nitrogen was determined automatically using a Buchi distillation and titration system (Buchi, Flawil, Switzerland). Protein values were determined by N(g) × 6.25 (AOAC, 1980). Ash was determined using the AOAC muffle furnace method (AOAC, 1980). Dehydrated imitation crab (≈2g) were heated in a muffle furnace (Lindberg, Model 51894, Watertown, WI) at 600°C for a minimum of 2 hr. Percent ash was calculated with adjustments to account for hydrated samples. Moisture content was determined using the AOAC (1980) vacuum oven method. Two replications of three samples from each treatment were dried at 77 mm Hg in a vacuum oven for 5 hr at 100°C. Carbohydrate was calculated by difference.

Sample pH was measured at room temperature (≈23°C) on two replications of duplicate samples from each treatment using a Corning pH meter (Model M103; Corning Medical and Scientific, Essex, England). pH values were determined using a method established for grading surimi quality (Lee, 1984; Lanier, 1992).

Sensory analysis

Sensory analysis was conducted in the sensory testing laboratory by 13–30 students, faculty, and staff for each of 10 panels. Training procedures for panel members generally followed those described by Meilgaard et al. (1991). Prior to sample testing, panelists were familiarized with attribute descriptions, anchor points and instructions relating to completion of descriptive ballots. Pretests were undertaken with selected samples of imitation crab to familiarize panelists with test samples as well as the measurement procedures.

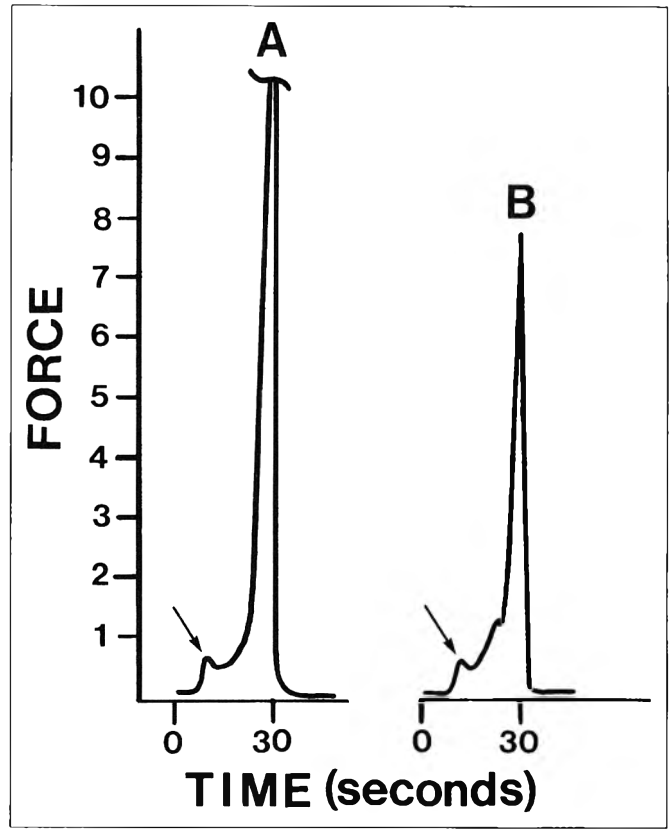

Fig. 1—Typical Instron compression curves of unheated (A = unheated control) and heated (B = baked 71) imitation crab legs. Points of fracturability (kg) for each curve are identified with an arrow. Hardness (kg) was determined by measuring height of the largest peak after 90% deformation of each sample.

Seated in individual booths lighted with standard fluorescent lighting (410 lux), each panelist received three half-leg samples of imitation crab which were served in covered glass petri dishes coded with random 3-digit numbers. A ballot consisting of unmarked 7-point linear scales for each attribute (Stone et al., 1974; 1980), room temperature water, and unsalted crackers were also provided. Panelists were allowed to evaluate the three samples in any order. Panelists evaluated each sample for chewiness/rubberiness, firmness, and color intensity. Firmness was defined as amount of force required to bite through the sample, and chewiness/rubberiness was defined as amount of work or effort the panelist had to exert in chewing to prepare the sample for swallowing. These definitions were adaptations of Szczesniak's textural definitions developed for use in sensory analysis (Szczesniak, 1963; Szczesniak et al., 1963). Color intensity was defined as visual perception of sample whiteness. A balanced incomplete block design was used for sample presentation to enable each of the 5 treatments to be tested with every other treatment at least once during the testing period (O'Mahony, 1986).

Statistical analysis

Two-way analysis of variance (ANOVA), analysis of covariance (ANCOVA) with interaction, and ANCOVA without interaction were applied to analyze data (Snedecor and Cochran, 1969; O'Mahony, 1986) using the GLM procedure (SAS Institute, Inc., 1985). F-ratios were used to determine significant differences at $P \leq 0.05$. Planned contrasts of interest were made between least square means of the unheated control and each of the four heat treated samples, between the 2 heating methods (baked and microwave heated), and between the two end point temperatures (71°C and 93°C). Data from Instron measures of texture, moisture, expressible moisture, and colorimeter measures were analyzed by ANOVA using means of each replication. Cross comparisons (Baked 71 vs Microwaved 93 and Baked 93 vs Microwaved 71) were not included.

Prior to statistical analysis, sensory data were decoded using a 7-point scale. All sensory data were weighted to account for variations in panel size. Time was also included in the statistical model to account for differences in length of time samples were in frozen storage between the first and last taste panels. Means of each taste panel were used for analysis, rather than individual judge scores, to compensate for variations in taste panel sizes.

RESULTS & DISCUSSION

Treatment effects on textural quality

Sensory data indicated that firmness and chewiness of room temperature samples were both affected by heating method but not by end point temperature (Table 2). Baked imitation crab samples were softer ($P < 0.05$) and less chewy ($P < 0.05$) than the two microwaved samples and the unheated control. Microwaved samples were also less firm ($P < 0.05$) and less chewy ($P < 0.05$) than unheated controls (Table 2).

In addition to treatments being significant by sample, statistical analysis indicated that a time by sample interaction for chewiness was significant ($P < 0.05$). Mean sensory scores for chewiness indicated that while chewiness gradually decreased over time in baked samples, this attribute gradually increased over time in microwaved and unheated control samples. Therefore, significant differences observed for time by sample interaction for chewiness at the average time of sensory testing (day 19) reflected those previously described for significance-by-sample data. No time by sample interaction occurred for any other sensory attribute.

Typical Instron compression curves illustrating hardness and point of fracture of heated and unheated imitation crab are shown (Fig. 1). Unheated imitation crab controls were firmer ($P < 0.05$) than all heated samples (Table 3). As with sensory data, these results were not associated with end-point temperature. Although slight variations in diameter of cooked samples may have influenced instrumental force measurements, magnitude differences for planned contrasts were assumed to be valid. The two methods showed heating consistently decreased firmness of imitation crab samples. Significant differences were not found among samples for fracturability, which suggested that attribute was unaffected by either heating method or end point temperature.

Many other studies have shown similarities between instrumental and sensory results. Sensory scores for firmness and chewiness have shown a high correlation with Instron shear and compressive force (Montejano et al., 1985; Lee et al., 1992b), as well as texturometer measures of hardness and chewiness (Szczesniak et al., 1963). Sensory attributes of firmness and chewiness correlated highly (Cardello et al., 1982).

Lee and Chung (1989) reported Instron penetration tests were the best measure of surimi gel firmness whereas Instron compression tests more accurately measured cohesiveness. However, they also found high correlations between compressive force and penetration force and both values correlated well with sensory firmness. Lee and Chung (1989), however, recommended compression tests over penetration tests to measure surimi firmness in samples which had been frozen and thawed.

The decrease in firmness and chewiness we found for heated imitation crab samples could have been caused by alterations in water-binding ability of starches during frozen storage (Lanier, 1986). Potato and wheat starches, 2 of the 3 starches in this product, produce strong, elastic gels when incorporated into surimi because of their water binding capacity (Lee, 1984; 1986; Lee and Kim, 1985; Kim and Lee, 1987; Lee et al., 1992a). However, such unmodified starches can readily retrograde, allowing syneresis during frozen storage. Consequently, a portion of the bound water which was released from cells could be expressed when thawed product is compressed or cut (Lee, 1986; Lee et al., 1992a).

Instron tests measuring expressible moisture revealed that unheated imitation crab controls contained ≈ 11% expressible moisture, whereas all heated samples contained <1% expressible moisture (Table 3). The high level of expressible moisture in unheated controls may be attributed to changes that occurred during frozen storage. Expressible moisture increased after one or more freeze-thaw cycles in surimi gels (Chung and Lee, 1991; Lee and Kim, 1985), fish gels (Cheng et al., 1979), kamaboko (Holmquist et al., 1984), and other surimi-based products (Lee, 1986; Lee et al., 1992a, b). Water expressed from starch by syneresis would not reassociate with starch after thawing and remained as free water in unheated samples (Whistler and Daniel, 1985). By comparison, starch in heated samples reversibly bound moisture, resulting in softer, less chewy products compared to unheated controls (Table 2). Similar findings in expressible moisture measurements have been reported in starches in surimi gel (Chung and Lee, 1991) and in imitation crab-like surimi products (Yoon and Lee, 1990).

Since moisture contents of baked and microwave heated samples were not different in moisture contents from unheated controls (Table 3), it is unlikely that expressible moisture differences were the result of moisture loss during heating. Furthermore, drip loss during thawing of samples was negligible. Firmer, chewier textures observed in unheated samples could be attributed to alignment of the starch matrix, which resulted in product toughening (Whistler and Daniel, 1985). Conversely, reassociation of water into the starch matrix resulted in greater water retention and, thus, decreased gel strength in heated samples. These results confirmed those reported earlier for surimi mixtures (Lee, 1984; Lee et al., 1992a), beef sausage mixtures (Gregg et al., 1993), gravies (Hanson et al., 1951), and reheated stale bread (Whistler and Daniel, 1985). Yoon and Lee (1990)

Table 2—Mean scores for the descriptive sensory analysis of imitation crab samples heat treated by either baking or microwave heating to an internal temperature of 71°C or 93°C and an unheated control

Sample description	Sensory attributes: Color intensity[d]	Firmness[e]	Chewiness/ Rubberiness[f]
	Least squares mean scores[g]		
Unheated control	3.66[a]	5.23[a]	5.16[a]
Baked 71	3.20[b]	3.44[b]	3.76[b]
Baked 93	3.62[a]	3.54[b]	3.78[b]
Microwaved 71	3.88[a,c]	4.14[c]	4.41[c]
Microwaved 93	3.95[c]	4.39[c]	4.44[c]

[a,b,c] For the planned contrasts only (see Materials and Methods), values in the same column with different superscripts are significantly different ($P < 0.05$).
[d] Scale: 1 = Very white; 7 = Brownish-white.
[e] Scale: 1 = Very soft; not firm; 7 = Extremely firm.
[f] Scale: 1 = Not chewy/rubbery; 7 = Extremely chewy/rubbery.
[g] n = 13-30.

Table 3—Hardness[c], fracturability[c], moisture, and expressible moisture[c] of baked, microwaved, and unheated control imitation crab samples

Sample description	Textural attributes: Hardness[e] (kg)	Fracturability[e] (kg)	Moisture (%)	Expressible moisture[d] (%)
	Mean values[f]			
Unheated Control	27.42[a]	0.57[a]	74.8[a,b]	11.31[a]
Baked 71	7.88[b]	0.53[a]	77.3[a]	0.87[b]
Baked 93	7.24[b]	0.57[a]	75.9[a,b]	0.81[b]
Microwaved 71	8.19[b]	0.52[a]	75.0[a,b]	0.70[b]
Microwaved 93	8.78[b]	0.49[a]	73.5[b]	0.76[b]

[a,b] For the planned contrasts only (see Materials & Methods), values in the same column with different superscripts are significantly different ($P < 0.05$).
[c] Compressed to 90% deformation by an Instron Universal Testing Machine using a 50 kg load cell.
[d] Expressible Moisture = Water Expressed/(Sample Weight × Moisture Content of Sample).
[e] Bourne (1978).
[f] Values reported reflect the mean of two replications of at least three samples from each treatment.

Table 4—Hunter color values and whiteness index reported for the exterior and interior of baked, microwaved, and unheated control samples of imitation crab

Sample description	Color indicator			
	Whiteness index[c]	L[d]	a[e]	b[f]
	mean values[g]			
Exterior Color				
Unheated Control	34.82[a]	71.56[a]	−2.80[a]	9.77[a]
Baked 71	39.95[a]	76.72[b]	−2.77[a]	9.49[a]
Baked 93	36.78[a]	73.88[a,b]	−2.79[a]	9.58[a]
Microwaved 71	38.53[a]	76.34[b]	−2.76[a]	9.85[a]
Microwaved 93	39.64[a]	74.95[b]	−2.78[a]	8.99[a]
Interior Color				
Unheated Control	40.89[a]	76.38[a]	−2.54[a]	9.29[a]
Baked 71	42.33[a]	77.53[a,b]	−2.78[b]	8.96[a]
Baked 93	41.57[a]	76.59[a]	−2.76[b]	8.91[a]
Microwaved 71	41.44[a]	77.87[b]	−2.70[a,b]	9.44[a]
Microwaved 93	41.71[a]	77.60[a,b]	−2.63[a,b]	9.33[a]

[a,b] For the planned contrasts only (see Materials and Methods), values in the same column within a treatment group with different superscripts are significantly different ($P < 0.05$).

[c] Whiteness index (WI): WI = L − 3b + 3a; (Gates and Parker, 1992). Used as an index of whiteness for blue crab.

[d] L Scale: Lightness/Darkness scale; white = 100; black = 0.

[e] a Scale: Red/green scale; red = (+); green = (−).

[f] b Scale: Yellow/blue scale; yellow = (+); blue = (−).

[g] Values reported reflect the mean of two replications of three samples from each treatment.

found that sensory texture scores and shear values declined when an imitation crab-like product containing starch and varying levels of added cellulose were reheated after undergoing three freeze-thaw cycles. However, when the same samples were subjected to three freeze-thaw cycles but not reheated, shear force values increased instead of decreasing.

In addition to textural changes, the degree of softening which occurred as a result of heating varied by heating method. Baked samples were softer and less chewy ($P < 0.05$) than microwaved samples, and similar results have been found in other foods (El-Shimi, 1992; Ream et al., 1974; Cruickshank, 1981; Rogers et al., 1990). In contrast, Madeira and Penfield (1985) found fish heated in a conventional oven was firmer and chewier than similar fillets heated by microwave. Kramer shear values for hardness gave similar results, and these values correlated highly with sensory panel scores for firmness and chewiness (Madeira and Penfield, 1985).

Although those studies used full microwave power to heat samples, in our study a reduced power level of 70% for sample heating was employed to achieve desired end point temperatures (Table 1). Previous studies resulted in conflicting results as to the effect of microwave power on product quality (Korschgen et al., 1976; Drew et al., 1980). Therefore, specific effects of microwave heating on textural characteristics of imitation crab are not clear.

Not all researchers have found heating method to have a greater influence on texture than end point temperature. Johansson et al. (1992) reported that tenderness of rainbow trout and cod were affected by end-point temperature to which samples were baked and microwave heated, but the method of heating did not affect tenderness. However, the highest temperature they used was about the same as the low temperature we used.

Treatment effects on color

Color appeared to be unrelated to heating method or end point temperature. Sensory panel scores found Baked 71 samples to be whiter ($P < 0.05$) than all other samples (Table 2). Baked 93 samples were more brownish-white ($P < 0.05$) than Baked 71 samples but whiter ($P < 0.05$) than Microwaved 93 samples.

Sensory scores indicated that imitation crab unheated controls were more brownish-white ($P < 0.05$) than Baked 71 samples, whiter ($P < 0.05$) than Microwaved 93 samples, and not different from Baked 93 or Microwaved 71 samples (Table 2). The microwaved samples were not different from each other. ANCOVA without interaction indicated that color differences discussed were significant not only by sample but also by time, although no time by sample interaction occurred.

The slight brownish-white color in Baked 93 samples probably resulted from Maillard browning. Interactions between fish protein amines, reducing sugars, and water during longer baking time and higher end point temperature of Baked 93 samples could occur (Whistler and Daniel, 1985). It is unlikely that Maillard reactions were responsible for browning of Microwaved 93 samples. Foods are less likely to brown when cooked by microwave heat because air inside the microwave oven remains too cool and moist during cooking to allow surface browning (Bakanowski and Zoller, 1984). The heating times we used would not have caused browning of microwaved samples.

Possibly some sample variations in whiteness resulted from differences in the rate at which starch rebound with expressible moisture of samples during heating. As starch granules imbibe more water, it changes from opaque (whiter) to more translucent (Charley, 1982). Heating to 71°C may have heated starch sufficiently to change the translucent unheated control to the more milky white starch binding with water. Continued heating to 93°C may have produced further color changes, resulting in the more translucent characteristics of gelatinized starch. Increased translucency could make the less white surimi color more obvious, giving the impression of decreased whiteness, and higher (less white) color scores.

Colorimetry measures of exterior Hunter L (black/white) values indicated that Baked 71, Microwaved 71, and Microwaved 93 imitation crab samples were whiter ($P < 0.05$) than unheated controls (Table 4). However, the three were not different from each other or from Baked 93 samples. No differences in whiteness as measured by L value existed between unheated controls and Baked 93 samples. Based on colorimeter mean L values, heating tended to result in whiter samples, regardless of heating method or end-point temperature.

Compared to sensory panel results, Hunter L values showed similar trends for whiteness for baked samples compared to unheated controls but different results for microwave heated samples compared to unheated controls. For baked samples, both sensory scores and colorimeter L values found Baked 71 samples were whiter ($P < 0.05$) than unheated controls. In addition, both sensory scores and Hunter L values showed no differences between unheated controls and Baked 93 samples. In contrast, Hunter L values indicated microwaved samples were whiter than unheated controls. Only Microwaved 93 samples vs unheated controls were less white ($P < 0.05$). No significant differences in exterior Hunter a (red/green) values, Hunter b (yellow/blue) values, or whiteness index were identified (Table 4).

Slightly different results were obtained with colorimetry measures of interior color (Table 4). Like exterior color, Hunter L values indicated Microwaved 71 samples were whiter ($P < 0.05$) than unheated controls, but no other differences were found. Only slight differences were found among samples for interior Hunter a values, and no differences were found among samples for interior Hunter b values or whiteness index (Table 4). Values for moisture (73.5–77.3%), lipid (0.3%), protein (11.5–13.5%), ash (2.0–2.5%), carbohydrate (8.8–10.2%), and pH (7.4) fell within ranges previously reported for surimi-based fabricated seafood products (Lee, 1986; Dudek et al., 1988).

CONCLUSIONS

HEATING DECREASED FIRMNESS and chewiness of imitation crab legs. The degree to which they were lessened related to the heating method used. Baking produced the softest and least chewy samples, microwave heating produced intermediate levels of firmness and chewiness, and no heating resulted in firmest and chewiest samples. End point temperature, however, had no significant effect on these attributes. Heating significantly de-

creased expressible moisture level of all four heated samples, regardless of heating method or end point temperature. Moisture contents of heated samples were not different from unheated controls. This suggests that baking and microwave heating caused free water (expressible moisture) to rebind with starch during heating.

REFERENCES

Anonymous. 1987. Surimi seafoods. Seafood Leader 7: 235–242.

AOAC. 1980. *Official Methods of Analysis*, 12th ed. Association of Official Analytical Chemists, Washington, DC.

Bakanowski, S.M. and Zoller, J.M. 1984. Endpoint temperature distributions in microwave and conventionally cooked pork. Food Technol. 38(2): 45–51.

Bligh, E.G. and Dyer, W.J. 1959. A rapid method of total lipid extraction and purification. Can. J. Biochem. Physiol. 37(8): 911–917.

Bourne, M.C. 1978. Texture profile analysis. Food Technol. 32(7): 62–66, 72.

Cardello, A.V., Sawyer, F.M., Maller, O., and Digman, L. 1982. Sensory evaluation of the texture and appearance of 17 species of North Atlantic fish. J. Food Sci. 47: 1818–1823.

Charley, H. 1982. *Food Science*, 2nd ed. John Wiley and Sons, New York.

Cheng, C.S., Hamann, D.D., Webb, N.B., and Sidwell, V. 1979. Effects of species and storage time on minced fish gel texture. J. Food Sci. 44: 1087–1092.

Chung, K.H. and Lee, C.M. 1991. Water binding and ingredient dispersion pattern effects on surimi gel texture. J. Food Sci. 56: 1263–1266.

Cruickshank, S.G. 1981. Texture of fish: A comparison of the effects of microwave and conventional heating. Master's thesis, Univ. of Maryland, College Park, MD.

Douglas-Schwarz, M. and Lee, C.M. 1988. Comparison of the thermal stability of red hake and Alaska pollock surimi during processing. J. Food Sci. 53: 1347–1351.

Drew, F., Rhee, K.S., and Carpenter, Z.L. 1980. Cooking at variable microwave power levels: effects on energy use and quality of top round beef roasts. J. Amer. Diet. Assoc. 77(4): 455–459.

Dudek, J.A., Elkins, E.R., and Chin, H.B. 1988. Investigation to determine the nutritional equivalency of surimi. p. 1–180. National Food Processors Association, Washington, DC.

El-Shimi, N.M. 1992. Influence of microwave and conventional cooking and reheating on sensory and chemical characteristics of roast beef. Food Chem. 45(1): 11–14.

Gates, K.W. and Parker, A.H. 1992. Characterization of minced meat extracted from blue crab picking plant by-products. J. Food Sci. 57: 267–270, 292.

Gregg, Jr., L.L., Claus, J.R., Hackney, C.R., and Marriott, N.G. 1993. Low-fat, high added water bologna from massaged, minced batter. J. Food Sci. 58: 259–264.

Hanson, H.L., Campbell, A., and Lineweaver, H. 1951. Preparation of stable frozen sauces and gravies. Food Technol. 5(10): 432–440.

Holmquist, J.F., Buck, E.M., and Hultin, H.O. 1984. Properties of Kamaboko made from red hake (*Urophycis chuss*) fillets, mince, or surimi. J. Food Sci. 49: 192–196.

Johansson, L., Ruderus, H., and Beilby, R.I. 1992. Optimum internal temperature established by sensory evaluation for fish prepared in conventional and microwave ovens. Home Econ. Res. J. 21(2): 192–205.

Kim, J.M. and Lee, C.M. 1987. Effect of starch on textural properties of surimi gel. J. Food Sci. 52: 722–725.

Korschgen, B.M., Baldwin, R.E., and Snider, S. 1976. Quality factors in beef, pork, and lamb cooked by microwaves. J. Amer. Diet. Assoc. 69(6): 635–640.

Lanier, T.C. 1986. Functional properties of surimi. Food Technol. 40(3): 107–114, 124.

Lanier, T.C. 1992. Measurement of surimi composition and functional properties. In *Surimi Technology*, T.C. Lanier and C.M. Lee (Ed.), p. 123–163. Marcel Dekker, Inc., New York.

Lanier, T.C., Lin, T.S., Liu, Y.M., and Hamann, D.D. 1982. Heat gelation properties of actomyosin and surimi prepared from Atlantic croaker. J. Food Sci. 47: 1921–1925.

Lee, C.M. 1984. Surimi process technology. Food Technol. 38(11): 69–80.

Lee, C.M. 1986. Surimi manufacturing and fabrication of surimi-based products. Food Technol. 40(3): 115–124.

Lee, C.M. and Chung, K.H. 1989. Analysis of surimi gel properties by compression and penetration tests. J. Texture Stud. 20: 363–377.

Lee, C.M. and Kim, J.M. 1985. Texture and freeze-thaw stability of surimi gels in relation to ingredients and formulation. In *Proceedings of the International Symposium on Engineered Seafood Including Surimi*, R.E. Martin and R.L. Collette (Ed.), p. 168–187. National Fisheries Institute, Washington, DC.

Lee, C.M., Wu, M.C., and Okada, M. 1992a. Ingredient and formulation technology for surimi-based products. In *Surimi Technology*, T.C. Lanier and C.M. Lee (Ed.), p. 273–302. Marcel Dekker, Inc., New York.

Lee, H.G., Lee, C.M., Chung, K.H. and Lavery, S.A. 1992b. Sodium ascorbate affects surimi gel-forming properties. J. Food Sci. 57: 1343–1347.

Madeira, K. and Penfield, M.P. 1985. Turbot fillet sections cooked by microwave and conventional heating methods: objective and sensory evaluation. J. Food Sci. 50: 172–177.

Meilgaard, M., Civille, G.V., and Carr, B.T. 1991. *Sensory Evaluation Techniques*, 2nd ed. CRC Press, Inc. Boca Raton, FL.

Montejano, J.G., Hamann, D.D., and Lanier, T.C. 1984. Thermally induced gelation of selected comminuted muscle systems—Rheological changes during processing, final strength and microstructure. J. Food Sci. 49: 1496–1505.

Montejano, J.G., Hamann, D.D., and Lanier, T.C. 1985. Comparison of two instrumental methods with sensory texture of protein gels. J. Texture Stud. 16(4): 403–424.

O'Mahony, M. 1986. *Sensory Evaluation of Food: Statistical Methods and Procedures*. Marcel Dekker, Inc., New York.

Ream, E.E., Wilcox, E.B., Taylor, F.G., and Bennett, J.A. 1974. Tenderness of beef roasts: microwave versus conventional cooking methods. J. Amer. Diet. Assoc. 65(2): 155–160.

Rogers, D.E., Doescher, L.C., and Hoseney, R.C. 1990. Texture characteristics of reheated bread. Cereal Chem. 67(2): 188–191.

SAS Institute, Inc. 1985. *SAS User's Guide: Statistics*. SAS Institute Inc., Cary, NC.

Shimizu, Y. 1985. Biochemical and functional properties of material fish. In *Proceedings of the International Symposium on Engineered Seafood Including Surimi*, R.E. Martin and R.L. Collette (Ed.), p. 148–167. National Fisheries Institute, Washington, DC.

Snedecor, G.W., and W.A. Cochran. 1969. *Statistical Methods*. Iowa State University Press, Ames, IO.

Stone, H., Sidel, J., Oliver, S., Woolsey, A., and Singleton, R.C. 1974. Sensory evaluation by quantitative descriptive analysis. Food Technol. 28(11): 24–34.

Stone, H., Sidel, J.L., and Bloomquist, J. 1980. Quantitative descriptive analysis. Cereal Foods World. 25(10): 642–644.

Szczesniak, A.S. 1963. Classification of textural characteristics. J. Food Sci. 28: 385–389.

Szczesniak, A.S., Brandt, M.A., and Friedman, H.H. 1963. Development of standard rating scales for mechanical parameters of texture and correlation between the objective and the sensory methods of texture evaluation. J. Food Sci. 28: 397–403.

Vondruska, J. 1985. Market trends and outlook for surimi-based foods. In *Proceedings of International Symposium on Engineered Seafood Including Surimi*, R.E. Martin and R.L. Collette (Ed.), p. 329–362. National Fisheries Institute, Washington, DC.

Whistler, R.L. and Daniel, J.R. 1985. Carbohydrates. In *Food Chemistry*, O.R. Fennema (Ed.). 2nd ed., p. 69–137. Marcel Dekker, Inc., New York.

Wu, M.C., Akahane, T., Lanier, T.C., and Hamann, D.D. 1985a. Thermal transitions of actomyosin and surimi prepared from Atlantic croaker as studied by differential scanning calorimetry. J. Food Sci. 50: 10–13.

Wu, M.C., Lanier, T.C., and Hamann, D.D. 1985b. Thermal transitions of admixed starch/fish protein systems during heating. J. Food Sci. 50: 20–25.

Wu, M.C., Lanier, T.C., and Hamann, D.D. 1985c. Rigidity and viscosity changes of croaker actomyosin during thermal gelation. J. Food Sci. 50: 14–19, 25.

Yoon, K.S. and Lee, C.M. 1990. Effect of powdered cellulose on the texture and freeze-thaw stability of surimi-based shellfish analog products. J. Food Sci. 55: 87–91.

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Alpha-Helical Structure of Fish Actomyosin: Changes during Setting

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ABSTRACT

Participation of the α-helix in setting was investigated using circular dichroism. The α-helicity of the actomyosin from eight species of fish decreased during incubation at 30°C or at 40°C. The extent and pattern of decrease differed among species. When rate of decrease was plotted vs rate of increase in strength of gel preincubated at 30°C or at 40°C, the two factors correlated closely. We propose that the unfolding of α-helix initiated setting.

Key Words: fish, actomyosin, circular dichroism, alpha helix

INTRODUCTION

WHEN SALTED FISH MEAT PASTE is kept at constant low temperature, its rheological properties change from sol to gel. This is called "setting." The setting response of fish meat paste differs with species (Shimizu et al., 1981). Setting has been attributed to hydrophobic interactions (Niwa, 1975; Niwa et al., 1981), the sulfhydryl-disulfide interaction (Itoh et al, 1979), and the covalent bond formation catalyzed by transglutaminase in the muscle (Seki et al., 1990; Kimura et al., 1991). Furthermore, the decrease in myosin heavy chain and a concomitant production of cross-linked myosin heavy chains were observed with setting (Numakura et al., 1985; Nishimoto et al., 1987, 1988; Niwa et al., 1989; Numakura et al., 1990; Nowsad et al., 1993). Apparently, setting is initiated during formation of bonds between myosin molecules. However, the mechanism of structural changes of myosin with setting has not been elucidated.

We reported that α-helical structure of fish myosin unfolded markedly in the temperature range 30 to 40°C, corresponding to high-temperature setting (Ogawa et al., 1993). Our current objective was to investigate the α-helical structural changes of actomyosin from several fish species to determine whether alterations of α-helical structure were associated with setting.

MATERIALS & METHODS

Materials

The 8 species of fish were: Amberjack (*Seriola dumerili*), Yellow Tail (*Seriola quinqueradiata*), Sea Bass (*Lateolabrax japonicus*), Sea Bream (*Pagrus major*), Greenling (*Hexagrammos otakii*), Stone Flounder (*Kareius bicoloratus*), Carp (*Cyprinus carpio*), Bigeye Tuna (*Thunnus obesus*). All species except Bigeye Tuna were used immediately post-mortem. Bigeye Tuna was used after storage on ice for 1 day post-mortem.

Preparation of actomyosin (AM)

AM was prepared according to the method of Sano *et al.* (1986) with modifications. Fish dorsal muscle (300g) was homogenized in 3,000 mL of 50 mM KCl-20 mM potassium phosphate buffer (pH 6.8) containing 0.01 mM (*p*-amidinophenyl) methanesulfonyl fluoride hydrochloride (*p*APMSF) in a Waring Blendor and the macerate was centrifuged at 4,500 × *g* for 20 min. The residue was homogenized in 3,000 mL of 0.6M KCl-20 mM potassium phosphate buffer (pH 7.0) in a Waring Blendor and the suspension was centrifuged at 4,500 × *g* for 20 min. The supernatant was filtered through triple-layer gauze. When the supernatant was diluted by adding 30 L of 20 mM potassium phosphate buffer (pH 6.8) with constant stirring, a precipitate formed. The precipitate was collected by centrifugation at 4,500 × *g* for 20 min and then suspended in 900 mL of 50 mM KCl-20 mM potassium phosphate buffer (pH 6.8). The washed precipitate was collected by centrifugation at 4,500 × *g* for 20 min. The residue was re-suspended in 600 mL of 50 mM KCl-20 mM potassium phosphate buffer (pH 6.8) containing 0.01 mM *p*APMSF. The purified AM was sedimented by centrifugation at 185,000 × *g* for 60 min.

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Preparation of gel

AM was adjusted to a protein concentration of 12% (w/w) with 50 mM KCl-20 mM potassium phosphate buffer (pH 6.8) and then mixed. The mixture was placed in a plastic bag, together with 2.5% NaCl (w/w), and ground for 5 min. The paste was deaerated by centrifugation at 1,800 × *g* for 10 min and added to a vinylidene chloride tube casing (31 mm in width) without introducing bubbles. The casing was sealed at both ends. The above procedures were performed at 4°C.

The samples were heated in a water bath in three regimes: at 90°C for 15 min (1) immediately after preparation, (2) after preincubation at 30°C for 60 min, and (3) after preincubation at 40°C for 30 min. Heated samples were cooled immediately in an ice-water bath for 30 min and held at 4°C for 24 hr.

Jelly strength

The jelly strength of the gel was measured by the puncture test using a rheometer (NRM-2010J-CW, Fudoh Kogyo Co., LTD., Tokyo, Japan) with a spherical plunger (5 mm in diameter). The plunger resting on the cylindrical gel sample (19 mm in diameter, cut to the length of 25 mm,) advanced at a speed of 6 cm/min. The breaking force (g) and the depth of the indentation (cm) were recorded. The product of the values (g × cm) was defined as jelly strength.

Circular dichroism (CD)

The supernatant after filtration through gauze was used as the sample for CD and diluted to a protein concentration of 0.2 mg/mL with 0.6M KCl-20 mM potassium phosphate buffer (pH 7.0). The CD measurement was carried out with a Jasco J-500A spectropolarimeter equipped with a water-jacketed cell holder. The temperature was controlled with a Thomas Kagaku Co., LTD Thomastat TRL-33. Ellipticities at 222 nm, $[\theta]_{222}$, were measured in 0.2-cm path length cell which was thermostated at 30°C or 40°C. α-Helicity was estimated by the following equation (Ogawa et al., 1993):

$$\alpha\text{-Helicity} = 100 \times \{[\theta]_{222} / -40{,}000\}$$

Protein concentration was determined by the modified biuret method of Umemoto (1966).

RESULTS & DISCUSSION

Changes in jelly strength of AM

The setting response was determined by the increase in jelly strength of AM (R_j) (Fig. 1). In Yellow Tail, Sea Bass, Greenling, and Bigeye Tuna, the jelly strength increased due to preincubation at 30°C, although the magnitude of the increase differed among species. In contrast, gels from Amberjack, Sea Bream, Stone Flounder, and Carp, showed a lowering of gel strength by preincubation. On the other hand, preincubation at 40°C caused a further increase in jelly strength in every species. Gels of AM from Sea Bream and Stone Flounder, although they

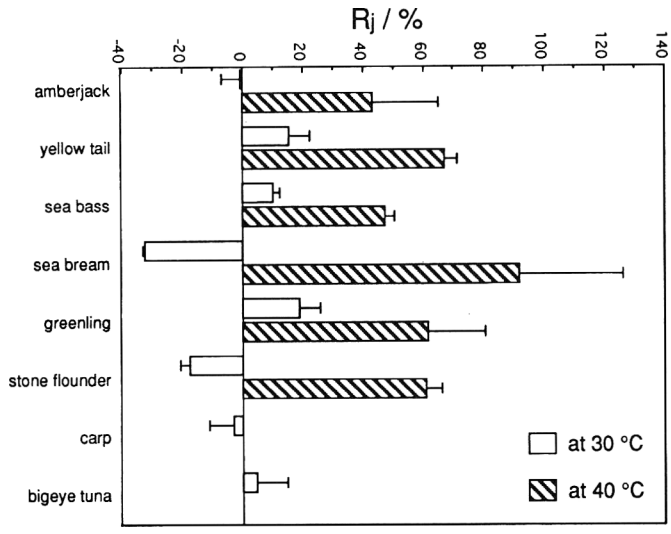

Fig. 1—Setting response of actomyosin. Rate of increase in jelly strength (R_j) (%) was expressed as 100 × {(the jelly strength of gel preincubated)/(the jelly strength of gel not preincubated) − 1}. (Averages of three independent measurements with standard deviations given by the error bars.)

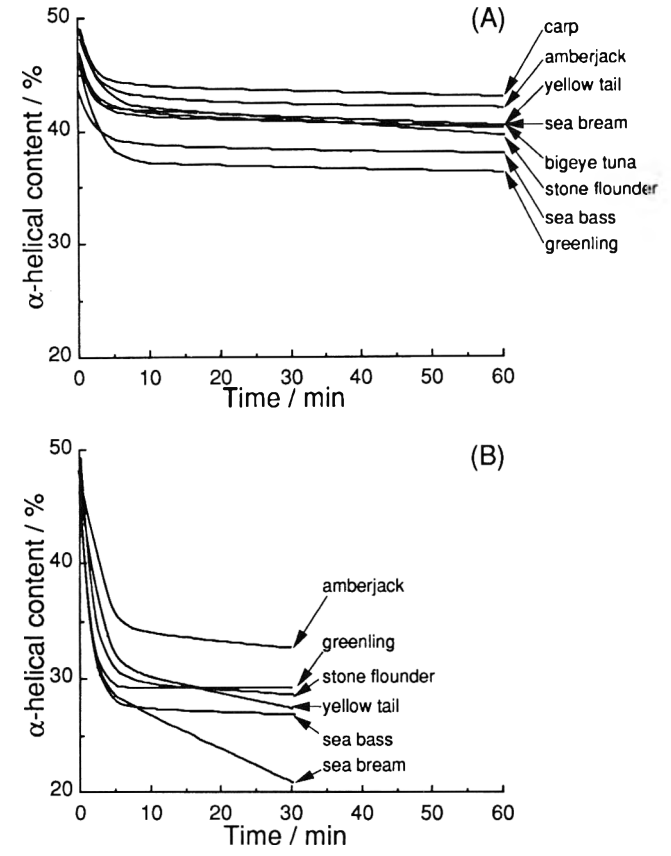

Fig. 2—Changes in α-helicity of actomyosin during incubation at (A) 30°C and (B) 40°C.

were weakened by preincubation at 30°C, were greatly strengthened by preincubation at 40°C. Thus, the setting response of fish AM differed not only among species, but between gel setting temperatures of 30°C and 40°C. These results were consistent with results of Shimizu et al. (1981).

Changes in α-helicity of AM

To elucidate the reason for such differences in setting, we investigated changes in the α-helicity of AM during incubation

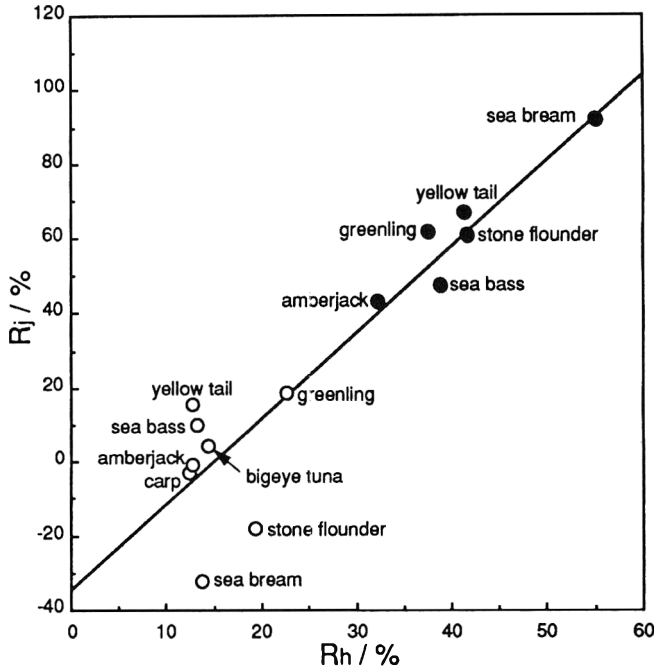

Fig. 3—Correlation between jelly strength (R_j) and rate of decrease in α-helicity (R_h). Values of R_j were averages in (Fig. 1). The R_h (%) was expressed as 100 × {(the α-helicity at incubation time of 60 min (30°C) or 30 min (40°C)/the α-helicity at incubation time of 0 min) − 1}. ○ at 30°C; ● at 40°C.

at 30°C or at 40°C (Fig. 2). In every species, at 30°C (Fig. 2A), the α-helicity decreased greatly in the first 5 min, but very little from 5 min to 60 min. The level of decrease during incubation of the AM solution for 60 min was 10% in Greenling and Stone Flounder, whereas it was only 6% in Yellow Tail and Carp. On the other hand, the α-helicity decrement at 40°C was still larger than that at 30°C for all AM samples. The decreasing α-helicity pattern at 40°C could be divided into two types (Fig. 2B). One type was similar to the pattern of AM samples heated at 30°C, and the other pattern showed that α-helicity continued to decrease after 5 min incubation time. The decrease from 5 min to 30 min was no less than 7% in Sea Bream. Accordingly, the extent and pattern of decrease at 40°C were different from those at 30°C. Since there were differences in α-helicity as well as in jelly strength between 30°C and 40°C, the possibility of a mutual relationship exists.

Comparison of gel and α-helicity

We examined whether setting was related to the decrease in α-helicity during incubation (R_h). The R_j increased with rise of R_h (Fig. 3). The correlation coefficient (r) between these was high (0.85). The strengthening of the gel may depend on the magnitude of unfolding of the α-helix. In less than 15% of the R_h, the regression line showed a negative R_j. Accordingly, it seems that R_h >15% enables jelly strength to increase. Interestingly, the higher R_j at 40°C of Sea Bream and Yellowtail might be related to the fact that the α-helix unfolding continued after 5 min. These reasons suggest that significant increase in jelly strength may require much unfolding over a long time at the temperature of preincubation.

Sano et al. (1990a,b) suggested that interactions among myosin tail portions with high α-helical content induced the initiation of gel elasticity. Such interactions may be due to unfolding of the tail portion during preincubation at 30°C or at 40°C. The formation of bonds initiated by unfolding would be essential to high levels of setting responses.

REFERENCES

Itoh, Y., Yoshinaka, R., and Ikeda, S. 1979. Effects of sulfhydryl reagents on the gel formation of carp actomyosin by heating. Nippon Suisan Gakkaishi 45: 1023–1025.

Kimura, I., Sugimoto, M., Toyoda, K., Seki, N., Arai, K., and Fujita, T. 1991. A study on the cross-linking reaction of myosin in kamaboko "suwari" gels. Nippon Suisan Gakkaishi 57: 1389–1396.

Nishimoto, S., Hashimoto, A., Seki, N., and Arai, K. 1988. Setting of mixed salted meat paste of two fish species in relation to cross-linking reaction of myosin heavy chain. Nippon Suisan Gakkaishi 54: 1227–1235.

Nishimoto, S., Hashimoto, A., Seki, N., Kimura, I., Toyoda, K., Fujita, T., and Arai, K. 1987. Influencing factors on changes in myosin heavy chain and jelly strength of salted meat paste from Alaska pollack during setting. Nippon Suisan Gakkaishi 53: 2011–2020.

Niwa, E. 1975. Role of hydrophobic bonding in gelation of fish fresh paste. Nippon Suisan Gakkaishi 41: 907–910.

Niwa, E., Sato, K., Suzuki, R., Nakayama, T., and Hamada, I. 1981. Fluorometric study of setting properties of fish flesh sol. Nippon Suisan Gakkaishi 47: 817–821.

Niwa, E., Yamada, H., Kanoh, S., and Nakayama, T. 1989. Thermal behavior of actomyosin subunits during setting of salted fish flesh sol. Nippon Suisan Gakkaishi 55: 1997–2000.

Nowsad, A.A., Kanoh, S., and Niwa, E. 1993. Electrophoretic behavior of cross-linked myosin heavy chain in suwari gel. Nippon Suisan Gakkaishi 59: 667–671.

Numakura, T., Kimura, I., Toyoda, K., and Fujita, T. 1990. Temperature-dependent changes in gel strength and myosin heavy chain of salt-ground meat from walleye pollack during setting. Nippon Suisan Gakkaishi 56: 2035–2043.

Numakura, T., Seki, N., Kimura, I., Toyoda, K., Fujita, T., Takama, K., and Arai, K. 1985. Cross-linking reaction of myosin in the fish paste during setting (suwari). Nippon Suisan Gakkaishi 51: 1559–1565.

Ogawa, M., Ehara, T., Tamiya, T., and Tsuchiya, T. 1993. Thermal stability of fish myosin. Comp. Biochem. Physiol. 106B: 517–521.

Sano, T., Noguchi, S.F., Matsumoto, J.J., and Tsuchiya, T. 1990a. Effect of ionic strength on dynamic viscoelastic behavior of myosin during thermal gelation. J. Food Sci. 55: 51–54 & 70.

Sano, T., Noguchi, S.F., Matsumoto, J.J., and Tsuchiya, T. 1990b. Thermal gelation characteristics of myosin subfragments. J. Food Sci. 55: 55–58 & 70.

Sano, T., Noguchi, S.F., Tsuchiya, T., and Matsumoto, J.J. 1986. Contribution of paramyosin to marine meat gel characteristics. J. Food Sci. 51: 946–950.

Seki, N., Uno, H., Lee, N., Kimura, I., Toyoda, K., Fujita, T., and Arai, K. 1990. Transglutaminase activity in Alaska pollack muscle and surimi, and its reaction with myosin B. Nippon Suisan Gakkaishi 56: 125–132.

Shimizu, Y., Machida, R., and Takenami, S. 1981. Species variations in the gel-forming characteristics of fish meat paste. Nippon Suisan Gakkaishi 47: 95–104.

Umemoto, S. 1966. A modified method for estimation of fish muscle protein by the biuret method. Nippon Suisan Gakkaishi 32: 427–435.

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Guadagni, D., Buttery, R., and Turnbaugh, J. 1972. Odor thresholds and similarity ratings of some potato chip components. J. Sci. Food Agric. 23: 1435–1444.

Hayashi, T., Yamaguchi, K., and Konosu, S. 1981. Sensory analysis of taste-active components in the extract of boiled snow crab meat. J. Food Sci. 46: 479–483.

Hsieh, T.C.-Y., Vejaphan, W., Williams, S.S., and Matiella, J.E. 1989. Volatile flavor components in thermally processed Louisiana red swamp crayfish and blue crab. Ch. 36, in *Thermal Generation of Aromas*, T.H. Parliment, R.J. McGorrin, and C.T. Ho (Ed.), p. 386–395. ACS Symposium Series 409, American Chemical Society, Washington, DC.

Jellinek, G. (Ed.). 1985. *Sensory Evaluation of Food: Theory and Practice*. VCH Publishers, Deerfield Beach, FL.

Josephson, D.B. and Lindsay, R.C. 1986. Enzymic generation of volatile aroma compounds from fresh fish. Ch. 17, in *Biogeneration of Aroma*, T.H. Parliment, R.J. McGorrin, and C.T. Ho. (Ed.), p. 386–395. ACS Symposium Series 409, American Chemical Society, Washington, DC.

Matiella, J.E. and Hsieh, T.C.-Y. 1990. Analysis of crabmeat volatile compounds. J. Food Sci. 55: 962–966.

Moncrieff, R.W. 1955. The sorptive properties of the olfactory membrane. J. Physiol. (London). 130: 543–?.

Morton, I.D., Akroyd, P., and May, C.G. 1960. Flavoring substances. U.S. Patent 2,934,437.

Mozell, M.M. 1970. Evidence for a chromatographic model of olfaction. J. Gen. Physiol. 56: 46–63.

Nunomura, N., Sasaki, M., Asao, Y., and Yokotsuka, T. 1976. Identification of volatile components in Shoyu (soy sauce) by gas chromatography-mass spectrometry. Agric. Biol. Chem. 40: 485–490.

Peers, K. and Swoboda, P.A.T. 1977. Volatile odorous compounds responsible for metallic, fishy taint formed in butterfat by selective oxidation. J. Sci. Food Agric. 28: 1010–1018.

Rayner, E.T., Dupuy, H.P., Legendre, M.G., Grodner, R.M., Cook, J.A., Novak, A.F., and Toloday, D.J. 1981. Instrumental analysis of seafood composition. J. Food Sci. 47: 76–78.

Roessler, E.B., Pangborn, R.M., Sidel, J.L., and Stone, H. 1978. Expanded statistical tables for estimating significance in paired-preference, paired-difference, duo-trio and triangle tests. J. Food Sci. 43: 940–944.

Schieberle, P. and Grosch, W. 1987. Evaluation of the flavour of wheat and rye bread crusts by aroma extract dilution analysis. Z. Lebensm. Unters. Forsch. 185: 111–113.

Swoboda, P.A.T. and Peers, K. 1977. Metallic odour caused by vinyl ketones formed in the oxidation of butterfat. The identification of octa-1-*cis*-5-dien-3-one. J. Sci. Food Agric. 28: 1019–1024.

van den Dool, H. and Kratz, P.D. 1963. A generalization of the retention index system including linear temperature programmed gas liquid partition chromatography. J. Chromatogr. 11: 463–471.

Wasserman, A.E. and Talley, F. 1968. Organoleptic identification of roasted beef, veal, lamb and pork as affected by fat. J. Food Sci. 33: 219–223.

Whitfield, F.B., Last, J.H., Shaw, K.J., and Tindale, C.R. 1988. 2,6-Dibromophenol: The cause of an iodoform-like off-flavour in some Australian Crustacea. J. Sci. Food Agric. 46: 29–42.

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Gel Strength Enhancement by Addition of Microbial Transglutaminase during Onshore Surimi Manufacture

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ABSTRACT

Surimi from Alaska pollock flesh was manufactured onshore with microbial transglutaminase (MTGase). Effect of MTGase was investigated by evaluating breaking strength and deformation of gels from MTGase-treated surimi with and without setting at 30°C. Quantitative analysis of ε-(γ-glutamyl)lysine (GL) crosslink was also carried out to monitor the MTGase reaction. In set gels, breaking strength and GL crosslink increased, and myosin heavy chain decreased correspondingly with MTGase concentration. These changes were smaller in gels prepared without setting. Results suggest that surimi gel could be improved through the formation of GL crosslinks by added MTGase in surimi.

Key Words: surimi, gel strength, transglutaminase, glutamyl-lysine

INTRODUCTION

THE TEXTURE OF FISH GEL PRODUCTS such as kamaboko and crab leg analog is an important quality which influences their preference and palatability. As a quality index of surimi, the strength of the gels has been evaluated.

To obtain a strong, elastic gel from surimi manufactured onshore has been very difficult. Only fresh, unfrozen fish can produce high-quality surimi and a strong gel. A decline in freshness and freeze-induced denaturation result in poor quality and weak gels. Land-grade surimi has undergone degradation by the time when it is made into surimi, and such degradation may further promote protein polymerization during frozen storage. As a result, protein lumps remain even after the kneading process. Such lumps apparently are condensed by heating and thus reduce the uniformity of the protein dispersion in the kamaboko, resulting in weakened product texture. Thus, manufacturing at sea has been required for high-quality surimi; however, the cost is higher compared with onshore surimi production (Lanier et al., 1992a; Sato and Tsuchiya, 1992).

In manufacturing fish gel products from surimi, several kinds of bonds, such as hydrophobic interactions, disulfide bonds, hydrogen bonds and others are formed (Niwa, 1992). Endogenous transglutaminases (TGases) in fish flesh may form ε-(γ-glutamyl)lysine (GL) crosslinks in fish proteins (Sato et al., 1992; Kumazawa et al., 1993a, b) and participate in textural changes of fish sol during processing (Seki et al., 1990; Tsukamasa and Shimizu, 1991; Wang et al., 1994). Tsukamasa et al. (1993) reported that the GL crosslink formed by such TGases was important in the low-temperature (25°C) setting of sardine flesh sol.

The activity of endogenous fish TGase and formation of GL crosslink in land-grade surimi was less when compared to those of factory ship-grade surimi (Kumazawa et al., 1993b). Increased formation of GL crosslink could reinforce the strength of land-grade surimi gel.

TGase is Ca^{2+}-dependent and catalyzes an acyl transfer reaction between the γ-carboxyamide group of glutamine residues of proteins and primary amines (Folk and Chung, 1973). When the primary amine is the ε-amino group of lysine residues of protein, GL crosslinks are formed. Although polymerization and crosslinking of some food proteins by mammalian TGases have been reported (Ikura et al., 1980a, b; Motoki and Nio, 1983; Kurth and Rogers, 1984), commercial utilization of such TGases in food production has scarcely been practiced due to high supply costs.

However, the situation changed after discovery of an extracellular microbial TGase (MTGase), produced by a variant of *Streptoverticillium mobaraense* (Ando et al., 1989; Washizu et al., 1994). A remarkable characteristic of that enzyme is its Ca^{2+}-independent catalytic property. Tanaka et al. (1990) reported polymerization and gelation of carp myosin with the MTGase. We hypothesized that the strength of the shore plant surimi gel would be improved by treatment with MTGase.

Our objective was to investigate the influence of added MTGase in onshore surimi manufacture and relationships between the GL crosslink formation by MTGase and the strength of gels made from MTGase-treated surimi.

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MATERIALS & METHODS

Preparation of surimi

Alaska pollock (*Theragra chalcogramma*) was harvested off the coast of Abasiri, and shipped on ice and processed to surimi within 2 days at an onshore factory in Abashiri city, Hokkaido, Japan. In brief, washed, strained and dewatered fish flesh (20 kg; moisture content 80%) was mixed for 7 min with a 6% (w/w of fish flesh) sucrose, 0.2% (w/w of fish flesh) sodium tripolyphosphate and appropriate amounts of MTGase (0–4.3 U/g protein). During mixing, the temperature was maintained below 7°C. The surimi product was frozen at −30°C. Moisture content of the surimi was 79.1%. Protein content was 14.5%, determined by Kjeldahl method (AOAC, 1990) using a conversion factor of 6.25.

Enzyme

MTGase (1.0 unit/mg) was prepared from the culture broth of a variant of *Streptoverticillium mobaraense* as previously described (Ando et al., 1989; Washizu et al., 1994). Enzymatic activity was measured by the hydroxamate procedure with Cbz-L-glutaminylglycine (Folk and Cole, 1966). Specific activity was defined as follows: 1 unit is that amount of enzyme which catalyzes the formation of 1 μmol hydroxamic acid/min at 37°C.

Preparation of surimi gels

Frozen surimi was tempered to −2°C and chopped in a silent cutter (12-kg type, Hanaki Seisakusho Co., Inc., Tokyo) for 9 min with sodium chloride (3.0% w/w), followed by chopping with potato starch (5.0% w/w) for 4 min. During chopping, the temperature was maintained below 10°C. The amount of salt and starch was based on the initial weight of the surimi. The surimi paste (~20g each) was stuffed into 30-mm-diameter polyvinylidene chloride tubes. Then the tubes were sealed and immersed in a water bath. The paste was incubated (set) for 60 min at 30°C followed by cooking for 30 min at 90°C. These gels were referred to as set (S) gels. For the control, the paste was directly cooked at 90°C for 30 min. These gels were referred to as non-set (NS) gels. After cooking, all gels were cooled in an ice-water bath for 10 min and stored in a room (25°C) overnight prior to instrumental measurements.

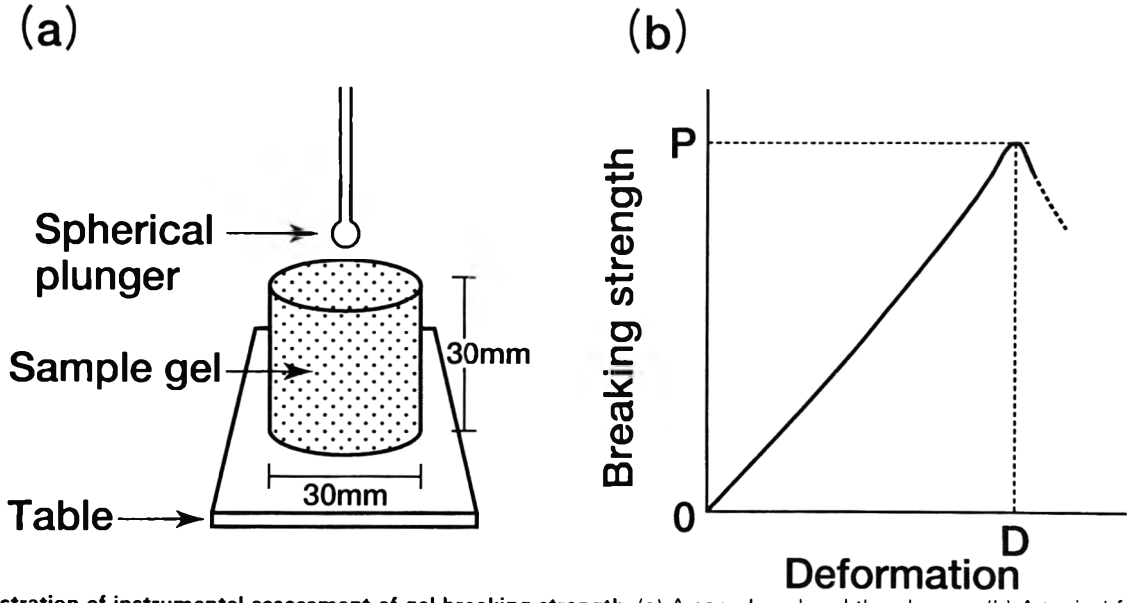

Fig. 1—Illustration of instrumental assessment of gel breaking strength. (a) A sample gel and the plunger. (b) A typical force versus deformation curve. P : breaking strength (g); D : deformation (cm).

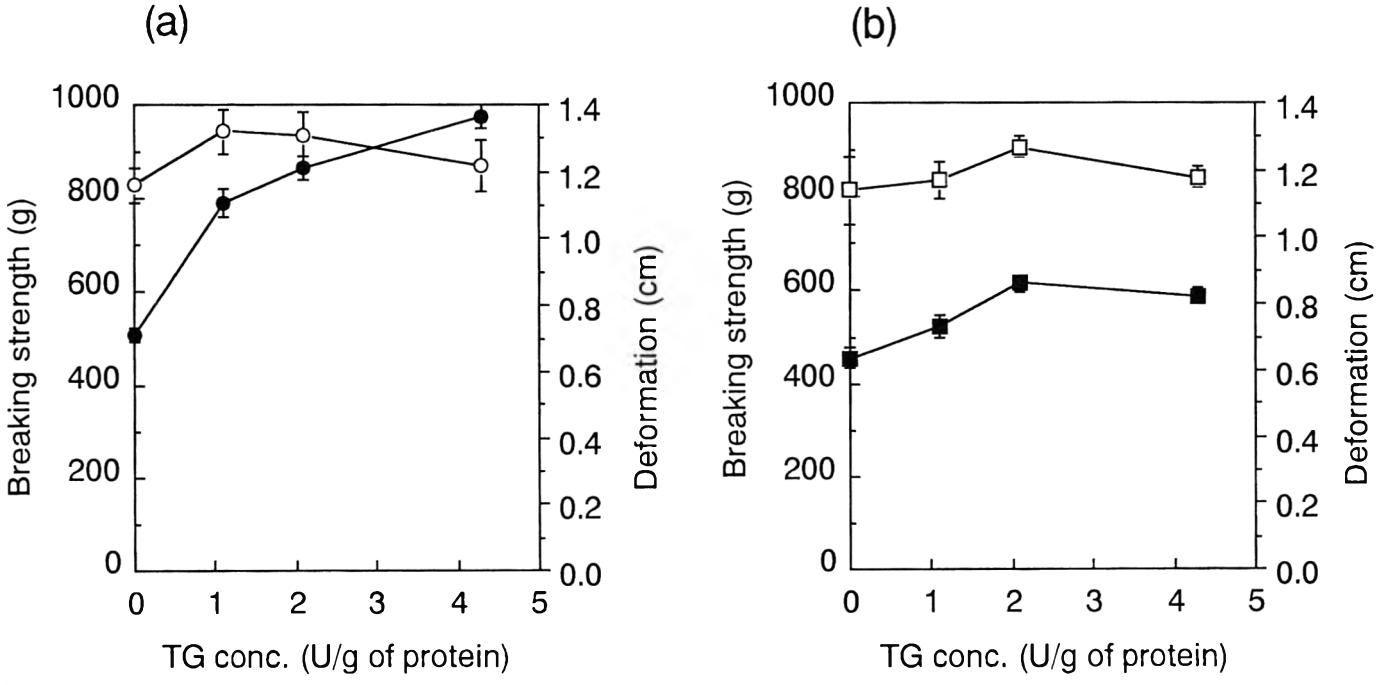

Fig. 2—Changes in breaking strength and deformation of surimi gels as a function of enzyme concentration. (a) S gels, set for 60 min at 30°C followed by cooking at 90°C for 30 min. ● breaking strength (g), ○ deformation (cm). (b) NS gels, cooked at 90°C for 30 min without setting. ■ breaking strength (g), □ deformation (cm).

Instrumental assessment of gel strength

The quality of the gels was assessed by measuring their breaking strength (g) and deformation (cm). A puncture test, previously described by Nonaka et al. (1992), was carried out with the gels at room temperature (25°C). Every sample piece was prepared to have 25 mm height by cutting the original cylindrically shaped gel. Each sample piece was set on the detachable table of a Rheometer™ (Fudo Kogyo K.K., Tokyo) equipped with a spherical plunger (ϕ = 5mm) (Fig. 1). Compression on the sample piece was executed with a table speed of 60 mm/min. The breaking strength was measured and read on the force vs. deformation curve by the value of the first force peak (g). The deformation was read on the same curve by dividing the sample height between the start point and the first peak force point (cm). Jelly strength was the multiplication of breaking strength by deformation (g × cm) (Lanier, 1992b).

The measurement was repeated 10 times on each sample gel, and the averages and standard deviations were calculated. To determine variance among those values which were observed as a function of enzyme concentrations, a one-way analysis of variance (one-way ANOVA) was performed (Ichihara, 1990; Ishihara et al., 1990).

SDS-polyacrylamide gel electrophoresis (PAGE) analysis

The gels were lyophilized and a small portion (8 mg) of each lyophilized gel was dissolved in 4 mL SDS solution, containing 2% SDS, 8M urea, 2% β-mercaptoethanol, 20 mM Tris-HCl (pH 8.0). The mixture was stirred for 20 hr at room temperature. An aliquot of solution was applied on SDS-PAGE gels (5–20% gradient gel) and electrophoresis was performed according to Laemmli's method (Laemmli, 1970). After the run, the SDS-PAGE gels were stained with Coomassie brilliant blue, and the myosin heavy chain content was determined by measuring the staining intensity using an UltroScan XL enhanced laser densitometer™

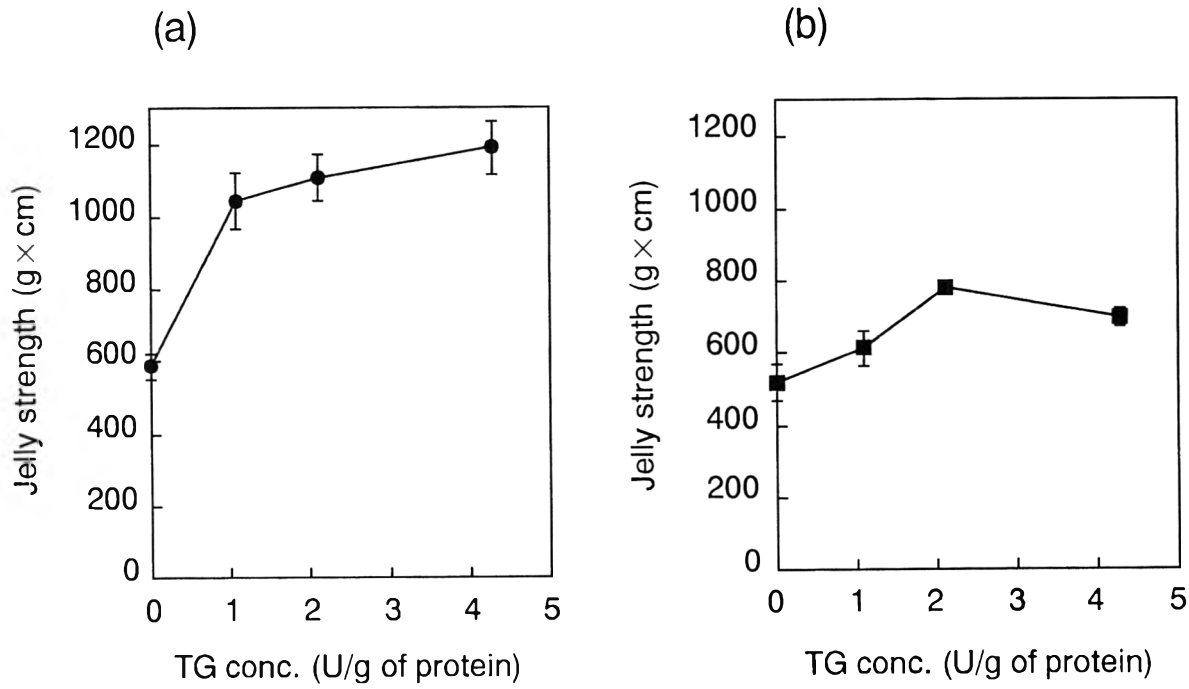

Fig. 3—Changes in jelly strength of surimi gels as a function of enzyme concentration. (a) S gels, set for 60 min at 30°C followed by cooking at 90°C for 30 min. (b) NS gels, cooked at 90°C for 30 min without setting.

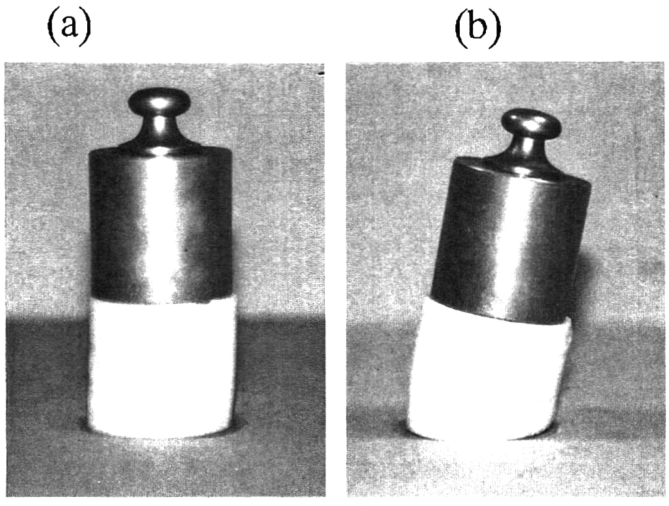

Fig. 4—Surimi gels. (a) S gel prepared from surimi with MTGase (4 U/g protein). (b) S gel prepared from surimi without MTGase. These S gels were set for 60 min at 30°C followed by cooking at 90°C for 30 min.

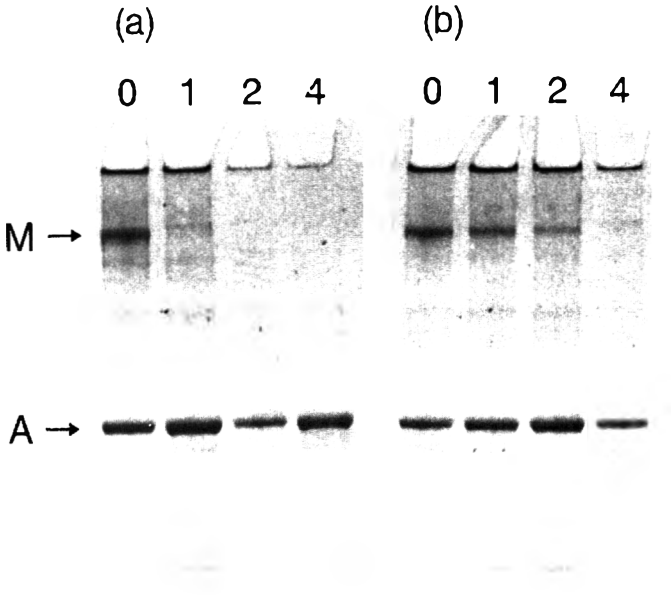

Fig. 5—SDS-PAGE pattern of myofibrillar proteins in surimi gels as related to enzyme concentration. Gels were dissolved in 8 M urea, 2% SDS, 2% β-mercaptoethanol, 20 mM Tris-HCl (pH 8.0) and then applied (10 μg protein/lane). The numbers above each lane indicate enzyme concentration (U/g protein). (a) S gels. (b) NS gels. Myosin heavy chain (M) and actin (A) are indicated by arrows.

(Pharmacia LKB Biotechnology, Uppsala, Sweden). The analysis was repeated 3 times on each sample and the average was calculated.

Analysis of ε-(γ-glutamyl)lysine

The gels were lyophilized and an aliquot (2 mL) of 0.1 M sodium horate buffer (pH 8.0) with a crystal of thymol was added to the protein sample (20 mg lyophilized powder). Sequential proteolytic digestion of this mixture and the GL content analysis were carried out as described elsewhere (Kumazawa et al. 1993a).

RESULTS

CHANGES IN BREAKING STRENGTH and deformation of the surimi gels (Fig. 2) showed breaking strength of S gels increased 90% as enzyme concentration increased. The deformation increased 13% at first when the enzyme was added at 1 U/g protein; however, it slightly decreased afterwards (Fig. 2a). In the case of NS gels, both breaking strength and deformation increased (35% on breaking strength, 10% on deformation) until the enzyme concentration reached 2 U/g protein, and a slight decrease occurred (Fig. 2b). The rate of the increment in breaking strength

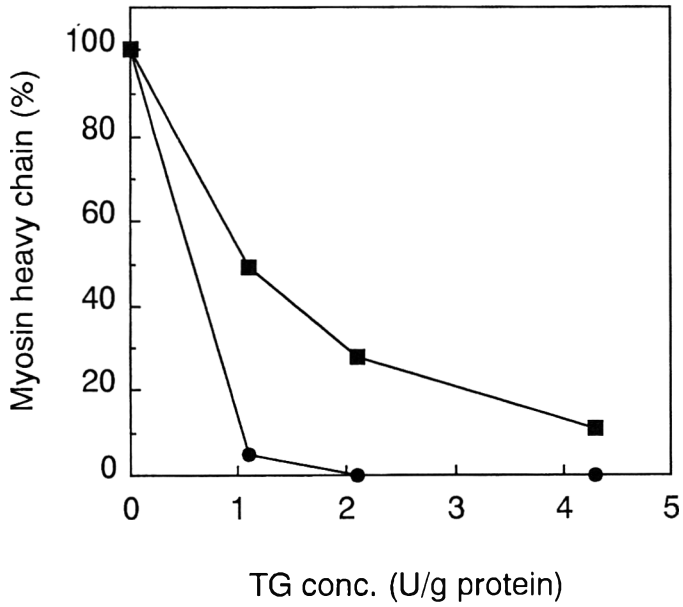

Fig. 6—Changes in myosin heavy chain content of myofibrillar protein as related to enzyme concentration. The myosin heavy chain content was estimated by quantitative densitometry using the gels in Fig. 5 ● S gel, ■ NS gel.

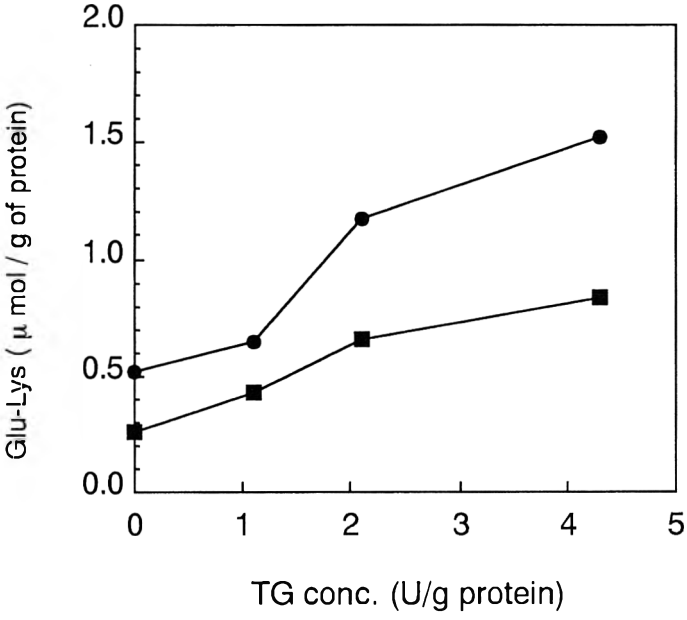

Fig. 7—Changes in ε-(γ-glutamyl)lysine content in surimi gels as related to enzyme concentration. Symbols and designations same as in Fig. 6.

for S gels was larger than that for NS gels. The relationship between MTGase concentration and jelly strength in S gels and NS gels (Fig. 3) showed the increment of jelly strength was obvious up to 1 or 2 U/g protein; however, excess of MTGase concentration had little effect or decreased jelly strength. All breaking strengths, deformations and jelly strengths were different as a function of enzyme concentrations ($P < 0.01$). In S gels. those with MTGase retained their original cylindrical shapes, even when a 200-g weight was loaded on. Those without MTGase did not retain the original shape with the weight (Fig. 4).

Changes in the SDS-PAGE pattern of myofibrillar proteins in both S and NS gels (Fig. 5) showed when the surimi paste was set at 30°C for 60 min, the band of myosin heavy chain apparently decreased with increased enzyme (Fig. 5a). Even when the surimi paste was not set, the decrease in the band of myosin heavy chain was observed (Fig. 5b). Such changes in myosin heavy chain were measured quantitatively by a densitometer. The myosin heavy chain content was expressed relative to the intensity of myosin heavy chain of the surimi gels prepared without MTGase (Fig. 6). In S gels, the myosin heavy chain content abruptly decreased as enzyme concentration increased and it finally approached zero. Since no bands appeared in the small-molecular-size region of the SDS-PAGE gels (Fig. 5a), such decrease in myosin heavy chain content may be ascribable to crosslinking of myosin heavy chain. A similar decrease in myosin heavy chain was observed in NS gels (Fig. 5b); however, the rate of decrease was smaller than that of S gels.

Formation of the GL crosslink in both S and NS gels was observed and the content increased as enzyme concentration increased to 4 U/g protein (Fig. 7). These data suggest that MTGase added into the surimi would generate GL crosslinks.

The rate of increase in GL content in S gels was larger than that in NS gels. The presence of the GL was observed even in S and NS gels prepared from surimi without MTGase (0 U/g protein on the abscissa). However, the increase in GL content proportionally increased with enzyme concentration. This indicates that it was the catalytic action of MTGase.

DISCUSSION

IN THE SURIMI GEL SYSTEM studied, the GL crosslink content in S gels was greater than in NS gels at all MTGase concentrations (Fig. 7). The difference between S and NS gels was in setting conditions, for NS gels were prepared without setting at 30°C for 60 min. Therefore, MTGase was thought to act during setting. The difference in preparative conditions would result in differences in breaking strengths between S and NS gels (Fig. 2). On the other hand, an apparent increase in GL content was observed in NS gels (Fig. 7). Ando et al. (1989) reported that purified MTGase lost 25% of its initial activity at 50°C for 10 min and we also observed that MTGase completely lost its activity at 60°C for 5 min (data not shown). However, the increase in GL content was likely to occur during cooking, since it is highly probable that MTGase catalyzed the reaction until it became totally inactivated during cooking. Coexisting proteins in the surimi affected the thermal stability of MTGase, or protected MTGase from heat inactivation. Evidence that a cooking condition at 63°C and 15 min failed to completely inactivate MTGase in surimi pastes (data not shown) support the results. Further, it was also likely that the GL crosslinks could be formed through the MTGase catalysis during the preparative process of surimi and/or surimi paste, even when the temperature was kept below 10°C.

As described (Fig. 3) the increments of jelly strength or the jelly strength decreased when more than 2 or 3 U/g protein were added. We hypothesized that excessive formation of GL crosslink would inhibit uniform development of the protein network and the improvement of breaking strength or deformation or jelly strength.

In several food protein gels, breaking strength was influenced by incubation time or temperature. In those gels, the increment of incubation time or temperature increased breaking strength. However, excessive time had no effect. Maximum gel breaking strengths were achieved at 50 or 65°C (Sakamoto et al., 1994). In kamaboko gels, the improvement of breaking strength or deformation by addition of MTGase was achieved at 10°C (Seguro et al., 1995). In this experiment the setting temperature was 30°C. At lower setting temperature, increments of breaking strength would be less or slower than at 30°C. However, at 50–70°C, modori (heat degradation) would occur, and those temperatures would not be effective for improvement of breaking strength (Matsumoto et al., 1992).

Formation of the GL crosslink by endogenous fish TGases in surimi has been reported (Sato et al., 1992; Kumazawa et al., 1993b; Tsukamasa et al., 1993). In gels prepared from surimi

manufactured without MTGase (0 U/g protein), the GL crosslink may be formed by such endogenous TGases in Alaska pollock. However, the endogenous TGase activity was lower (Kumazawa et al., 1993b) when compared to those with added MTGase and, thus, the resulting GL content was also smaller in that case.

The decrease in myosin heavy chain (Fig. 6) correlated with the increase in GL crosslink content (Fig. 7). Similarly, the increase in breaking strength was thought to be influenced by the formation of the GL crosslinks. Kimura et al. (1991) reported that water-soluble protein components of hoki (*Macruronus novaezelandiae*) surimi catalyzed the crosslinking reaction of myosin heavy chain and resulted in kamaboko by setting procedures. They demonstrated that formation of GL crosslinks was found during setting of surimi gel. However, they did not carry out quantitative analysis of GL crosslinks. Tsukamasa et al. (1993) reported the relationship between formation of GL crosslinks and gel strength of sardine gel with quantitative analysis. They demonstrated that gel strength increased as GL content increased in the low-temperature setting.

Gels prepared in our study were cooked during the last stage as are commercial surimi products. The breaking strength was thought to be influenced by several bonds besides GL crosslinks. Extensive studies have revealed that hydrogen bonds, hydrophobic interactions and disulfide bridges are important in determining the elasticity, firmness and other physical properties of surimi-based products (Niwa 1992). However, covalent crosslinking reactions other than disulfide bridges have been reported to be involved in setting (Seki et al., 1990; Kimura et al., 1991; Tsukamasa and Shimizu, 1991).

CONCLUSION

GL CONTENT increased with an increase in MTGase concentration, and breaking strength increased with increase in GL content. A correlation between breaking strength and GL crosslinks was observed. Addition of MTGase during onshore surimi manufacture was effective in increasing gel strength. Our results are applicable to present surimi production and processing of fish gel products. The manufacturing of surimi with MTGase may improve the quality of surimi gel products.

REFERENCES

Ando, H., Adachi, M., Umeda, K., Matsuura, A., Nonaka, M., Uchio, R., Tanaka, H., and Motoki, M. 1989. Purification and characteristics of a novel transglutaminase derived from microorganisms. Agric. Biol. Chem. 53: 2613–2617.

AOAC 1990. *Official Methods of Analysis*. 15th ed. Association of Official Analytical Chemists, Washington, DC.

Folk, J.E. and Chung, S.I. 1973. Molecular and catalytic properties of transglutaminases. Adv. Enzymol. 38: 109–191.

Folk, J.E. and Cole, P.W. 1966. Mechanism of action of guinea pig liver transglutaminase. J. Biol. Chem. 241: 5518–5525.

Ichihara, K. 1990. *Statistics for Bioscience—Practical Technique and Theory*. Nankodo, Tokyo, Japan.

Ikura, K., Kometani, T., Sasaki, R., and Chiba, H. 1980a. Crosslinking of soybean 7S and 11S proteins by transglutaminase. Agric. Biol. Chem. 44: 2979–2984.

Ikura, K., Kometani, T., Yoshikawa, M., Sasaki, R., and Chiba, H. 1980b. Crosslinking of casein components by transglutaminase. Agric. Biol. Chem. 44: 1567–1573.

Ishihara, T., Hasegawa, K., and Kawaguchi, T. 1990. *An Introduction of Lotus 1-2-3 to Multivariate Statistical Analysis*. Kyoritsu syuppan, Tokyo Japan.

Kimura, I., Sugimoto, M., Toyoda, K., Seki, N., Arai, K., and Fujita, T. 1991. A study on the cross-linking reaction of myosin in kamaboko "suwari" gels. Nippon Suisan Gakkaishi 57: 1389–1396.

Kumazawa, Y., Seguro, K., Takamura, M., and Motoki, M. 1993a. Formation of ε-(γ-glutamyl)lysine cross-link in cured horse mackerel meat induced by drying. J. Food Sci. 58: 1086–1089.

Kumazawa, Y., Numazawa, T., Seguro, K., Takamura, M., and Motoki, M. 1993b. Participation of transglutaminase in the manufacturing process of "kamaboko" Paper no. 124A, presented at the 53rd Annual Meeting of the Inst. of Food Technologists, Chicago, IL, July 10–14.

Kurth, L. and Rogers, P.J. 1984. Transglutaminase-catalyzed cross-linking of myosin to soya protein, casein and gluten. J. Food Sci. 49: 573–589.

Laemmli, U.K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227: 680–685.

Lanier, T.C., 1992b. Measurement of surimi composition and functional properties. In *Surimi Technology*, T.C. Lanier and C.M. Lee (Ed.), p. 123–163. Marcel Dekker, Inc., New York.

Lanier, T.C., Manning, P.K., Zetterling, T., and MacDonald, G.A. 1992a. Process innovations in surimi manufacture. In *Surimi Technology*, T.C. Lanier and C.M. Lee (Ed.), p. 167–179. Marcel Dekker, Inc., New York.

Matsumoto, J.J. and Noguchi, S.F. 1992. Cryostabilization of protein in surimi. In *Surimi Technology*, T.C. Lanier and C.M. Lee (Ed.), p. 357–388. Marcel Dekker, Inc., New York.

Motoki, M. and Nio, N. 1983. Crosslinking between different food proteins by transglutaminase. J. Food Sci. 48: 561–566.

Niwa, E. 1992. Chemistry of surimi gelation. In *Surimi Technology*, T.C. Lanier and C.M. Lee (Ed.), p. 389–427. Marcel Dekker, Inc., New York.

Nonaka, M., Sakamoto, H., Toiguchi, S., Kawajiri, H., Soeda, T., and Motoki, M. 1992. Sodium caseinate and skim milk gels formed by incubation with microbial transglutaminase. J. Food Sci. 57: 1214–1218.

Sakamoto, H., Kumazawa, T., and Motoki, M. 1994. Strength of protein gels prepared with microbial transglutaminase as related to reaction conditions. J. Food. Sci. 59: 866–871.

Sato, A., and Tsuchiya, T. 1992. Microstructure of surimi and surimi-based products. In *Surimi Technology*, T.C. Lanier and C.M. Lee (Ed.), p. 501–518. Marcel Dekker, Inc., New York.

Sato, K., Tsukamasa, Y., Imai, C., Ohtsuki, K., Shimizu, Y., and Kawahata, M. 1992. Improved method for identification and determination of ε-(γ-glutamyl)lysine cross-link in protein using proteolytic digestion and derivatization with phenyl isothiocyanate followed by high-performance liquid chromatography separation. J. Agric. Food Chem. 40: 806–810.

Seguro, K., Kumazawa, Y., Ohtsuka, T., and Motoki, M. 1995. Microbial transglutaminase and ε-(γ-glutamyl) lysine crosslinks effects on elastic properties of kamaboko gels. J. Food Sci. 60(2).

Seki, N., Uno, H., Lee, N.-H., Kimura, I., Toyoda, K., Fujita, T., and Arai, K. 1990. Transglutaminase activity in Alaska pollock muscle and surimi, and its reaction with myosin B. Nippon Suisan Gakkaishi 56: 125–132.

Tanaka, H., Nonaka, M., and Motoki, M. 1990. Polymerization and gelation of carp myosin by microbial transglutaminase. Nippon Suisan Gakkaishi 56: 1341.

Tsukamasa, Y. and Shimizu, Y. 1991. Factors affecting the transglutaminase-associated setting phenomenon in fish meat sol. Nippon Suisan Gakkaishi 57: 535–540.

Tsukamasa, Y., Sato, K., Imai, Shimizu, Y., Imai, C., Sugiyama, M., Minegishi, Y., and Kawabata, M. 1993. ε-(γ-Glutamyl)lysine crosslink formation in sardine myofibril sol during setting at 25°C. J. Food Sci. 58: 785–787.

Wang, J., Kimura, I., Satake, M., and Seki, N. 1994. Effect of calcium ion concentration on the gelling properties and transglutaminase activity of walleye pollack surimi paste. Fisheries Science 60: 107–113.

Washizu, K., Ando, K., Koikeda, S., Hirose, S., Matsuura, A., Takagi, H., Motoki, M., and Takeuchi, K. 1994. Molecular cloning of the gene for microbial transglutaminase from *Streptoverticillium* and its expression in *Streptomyces lividans*. Biosci. Biotech. Biochem. 58: 82–87.

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Microbial Transglutaminase and ε-(γ-Glutamyl)lysine Crosslink Effects on Elastic Properties of Kamaboko Gels

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ABSTRACT

Kamaboko gels from Alaska pollock surimi (SA™ and 2nd™ grades) were prepared by setting at 10 or 45°C with microbial transglutaminase (MTGase) and its effect on gel properties was investigated. At 10 and 45°C, gels from 2nd™ grade surimi paste showed increases in breaking strength, without decline in deformation. Gel from SA™ graded surimi paste showed an increase in breaking strength with no changes in deformation in 45°C setting, up to 0.03% MTGase. Crosslinking of myosin heavy chains through ε-(γ-glutamyl)lysine bonds was observed and a possible correlation was shown between ε-(γ-glutamyl)lysine content and gel strength (breaking strength X strain). ε-(γ-Glutamyl)lysine content up to 3 μmol/100g or MTGase 0.03% or higher improved gel properties.

Key Words: surimi, transglutaminase, viscoelasticity, glutamyl-lysine crosslink

INTRODUCTION

GEL-FORMING ABILITY and viscoelastic property (*ashi*) are important for surimi and surimi-based products, particularly kamaboko (Shimizu, 1981, 1987; Urch, 1988; Hamann, 1992; Lanier et al., 1992). These properties are largely dependent on modes of interactions and bonds, such as hydrogen bonds electrostatic and hydrophobic interactions. These and disulfide bonds and ε-(γ-glutamyl)-lysine (ε-(γ-Glu)Lys) crosslink (Wicker et al., 1989; Niwa, 1992) have been hypothesized to be formed through the phasic change from surimi sol to gel during the manufacturing process. Among such bonds, ε-(γ-Glu)Lys crosslink is covalent and may be important in gel-forming and viscoelastic properties. And its effect in fish flesh is reportedly to impart viscoelastic properties to surimi-based products (Kimura et al., 1991; Tsukamasa and Shimizu, 1991; Kamath et al., 1992; Sato et al., 1992; Tsukamasa et al., 1993; Wang et al., 1994).

ε-(γ-Glu)Lys crosslink is a catalytic product of transglutaminases (TGases) formed between the γ-carboxyamide group of peptide-bound glutamine residues and ε-amino group of peptide-bound lysine residues or free lysine. TGases have been found not only in fish flesh, but also in microorganisms, vegetables, cattle, shellfish and humans (Folk and Chung, 1973; Folk and Finlayson, 1977; Folk, 1980; Kurth and Rogers, 1984; Icekson and Apelbaum, 1987; Margosiak et al., 1990; Ramanujam and Hageman, 1990; Seki et al., 1990; Kishi et al., 1991; Klein et al., 1992; Signorini et al., 1991; Araki and Seki, 1993; Kumazawa et al., 1993b). TGase has also been found in a culture broth of a variant of *Streptoverticillium mobaraense* (Ando et al., 1989; Washizu et al., 1994) and referred to as MTGase. The enzyme catalyzes the same reaction, and polymerized and gelled carp and rabbit myosins (Nonaka et al., 1989; Tanaka et al., 1990). Therefore, we hypothesized that the gel-forming and viscoelastic properties of surimi-based products would be improved by a treatment with MTGase.

Our objective was to investigate the effect of MTGase addition on kamaboko manufacturing both at low- and high-temperature preincubations, and to check correlations between gel strengths of MTGase-treated gels and ε-(γ-Glu)Lys crosslink contents resulting from the MTGase treatment.

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MATERIALS & METHODS

Chemicals

Salt (NaCl) and most reagents were of guaranteed grade and were purchased from Wako Pure Chem. Co. (Osaka, Japan). Amino acid standard mixtures (Types H; a mixture of 17 amino acids and ammonium chloride), trifluoroacetic acid (TFA), acetonitrile, tetrahydrofuran (THF), potassium acetate, o-phthalaldehyde (OPA) were of analytical or protein-sequencing grade and were also purchased from Wako Pure Chemicals Co. Synthetic ε-(γ-Glu)Lys and glutamic acid γ-monohydroxamate were purchased from Sigma Chem. Co. (St. Louis, MO).

Transglutaminase

MTGase (1.0 unit/mg) was prepared from the culture broth of a variant of *Streptoverticillium mobaraense* as previously described (Ando et al., 1989; Washizu et al., 1994). Enzymatic activity was measured by the hydroxamate procedure with carbobenzyloxy-L-glutaminylglycine as a substrate (Folk and Cole, 1966). In this measurement, the substrate was transformed into a γ-glutamyl-hydroxamate form by the enzyme and then into a complex with ferric ion in acidic conditions, with maximum absorbance at 525 nm. The enzymatic activity was estimated from readings at 525 nm and the calibration curve drawn with glutamic acid γ-monohydroxamate as standard. The specific activity was defined as follows: 1 unit is the amount of enzyme which catalyzes the formation of 1 μmol hydroxamic acid/min at 37°C.

Proteolytic enzymes

Pronase from *Streptomyces griseus* and carboxypeptidase A from bovine pancreas were purchased from Boehringer Mannheim GmbH (Germany). Leucine aminopeptidase and prolidase from porcine kidney were purchased from Sigma.

Preparation of surimi gels

Frozen surimi (SA™ and 2nd™ grades) of Alaska pollock (*Theragra chalcogramma*) was purchased from Maruha Co. (Tokyo) and 250g each was tempered to −2°C and chopped in a speed cutter (Model MK-K3, Matsushita Electric Industrial Co. Ltd., Japan) for 5 min, followed by chopping with salt (3.0% w/w) and water (20% w/w) for 4 min. The amount of salt and water was based on initial weight of the surimi. The temperature was maintained below 2°C during chopping. The surimi paste was stuffed into 30-mm-diameter polyvinylidene chloride tubes. Then the tubes were sealed and immersed in a water bath. In the experiment with MTGase concentration as a variable, MTGase (0.01–0.07% (w/w)) was dissolved in the water and added as solution. Then the pastes in the tubes were preincubated ("set") at either 10°C for 16 hr or 45°C for 30 min, prior to cooking at 85°C for 30 min. In the experiment with setting time as a variable, the pastes with 0.03% MTGase added were set at either 10°C or 45°C for up to 32 hr or 60 min, respectively, followed by cooking at 85°C for 30 min. The gels set at 10°C were referred to as L gels and those set at 45°C as H gels. And designations "/A" and "/2" after H or L indicate that gels were made from SA™ and 2nd™ grade surimi pastes, respectively. For the control, gels with no MTGase added were similarly treated. After cooking at 85°C, all the gels were cooled in an ice-water bath for 10 min and stored in a room (25°C) overnight prior to instrumental measurements.

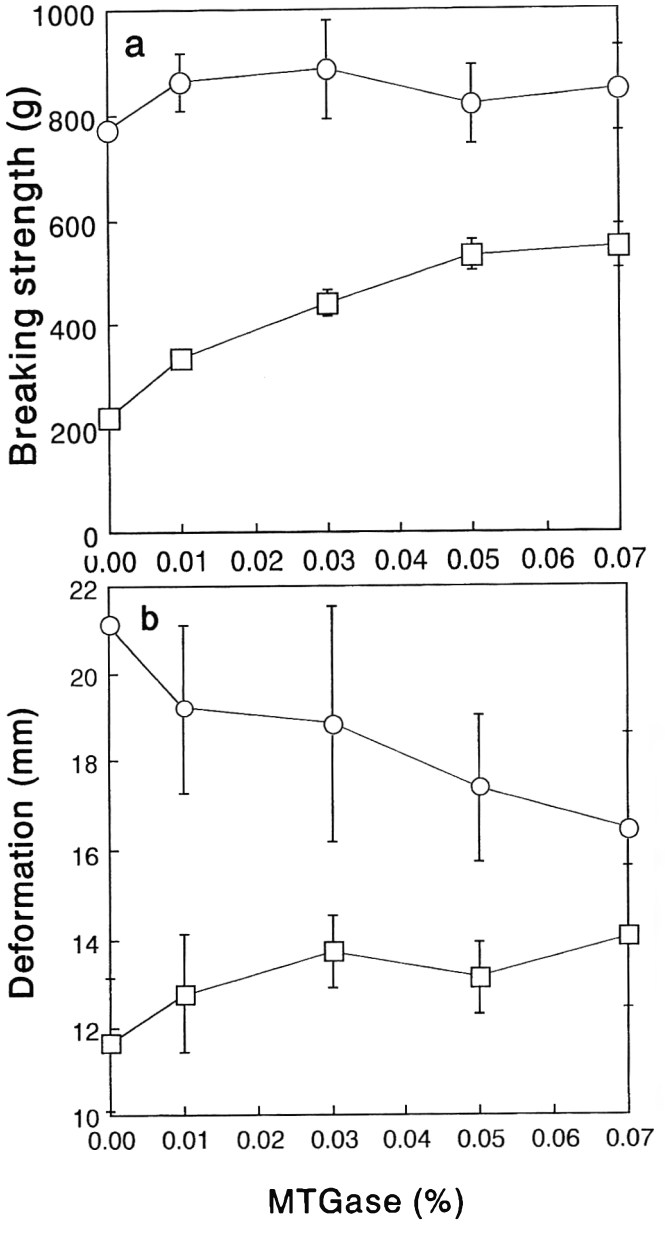

Fig. 1—Changes in breaking strength and deformation of kamaboko gels set at 10°C for 16 hr as related to MTGase concentration. L/A gel, ○; L/2 gel, □. Vertical bars represent Standard deviation. For details, see Materials & Methods.

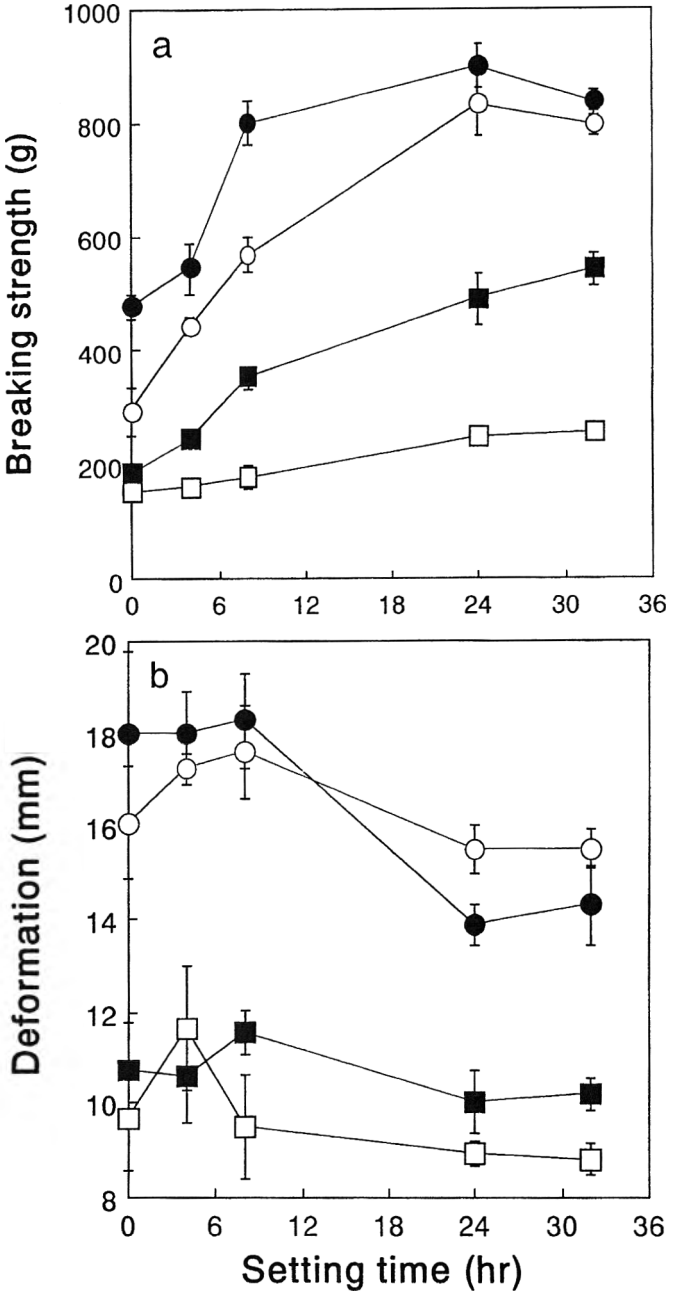

Fig. 2—Changes in breaking strength and deformation of kamaboko gels set at 10°C as related to setting time. L/A gel, ○ ●; L/2 gel, □ ■. Gels with MTGase added, filled symbols; gels without MTGase, open symbols. Vertical bars represent Standard deviation. For details, see Materials & Methods.

Instrumental assessment of jelly strength

The quality of the gels was assessed by measuring their breaking strength (g) and deformation (mm). A puncture test, as previously performed by Nonaka et al. (1992), was also carried out with a 5-mm spherical plunger at room temperature (25°C) in this study. The breaking strength was obtained from a force vs. deformation curve of the first force peak. The measurement was repeated 4 times on 2 pieces from each gel, and the average was calculated. Jelly strength was obtained by multiplying the breaking strength and deformation.

SDS-polyacrylamide gel electrophoresis (PAGE) analysis

The gels were lyophilized and a portion (20 mg) from each lyophilized gel was dissolved in 2 mL SDS-urea solution, containing 2% SDS, 8M urea, 2% β-mercaptoethanol, 20 mM Tris-HCl (pH 8.0) as described by Numakura et al. (1985). The mixture was heated at 100°C for 2 min and afterwards stirred for 20 hr at room temperature (≈23°C). An aliquot of the solution was applied to SDS-PAGE gels (5–20% gradient gel) and electrophoresis was performed according to Laemmli's method (Laemmli, 1970) with a low-molecular-weight standard (Pharmacia). After the run, the SDS-PAGE gels were stained with Coomassie brilliant blue, and the myosin heavy chain (MHC) content was determined by measuring the staining intensity of MHC using an enhanced laser densitometer (UltroScan XL™, Pharmacia LKB Biotechnology, Uppsala, Sweden). The analysis was repeated 3 times on each sample and the average was calculated.

Analysis of ε-(γ-Glu)Lys

The gels, cut into small pieces, were lyophilized and an aliquot (2 mL) of 0.1M sodium borate buffer (pH 8.0), with a crystal of thymol as a preservative, was added to the lyophilized protein sample (30 mg) in a plastic test tube. Sequential proteolytic digestion of this mixture with pronase, leucine aminopeptidase, prolidase and carboxypeptidase A was done exhaustively. A two-step fractionation of ε-(γ-Glu)Lys with high-performance liquid chromatography (HPLC) was carried out and its content in the gels was determined as previously described (Kumazawa et al., 1993a).

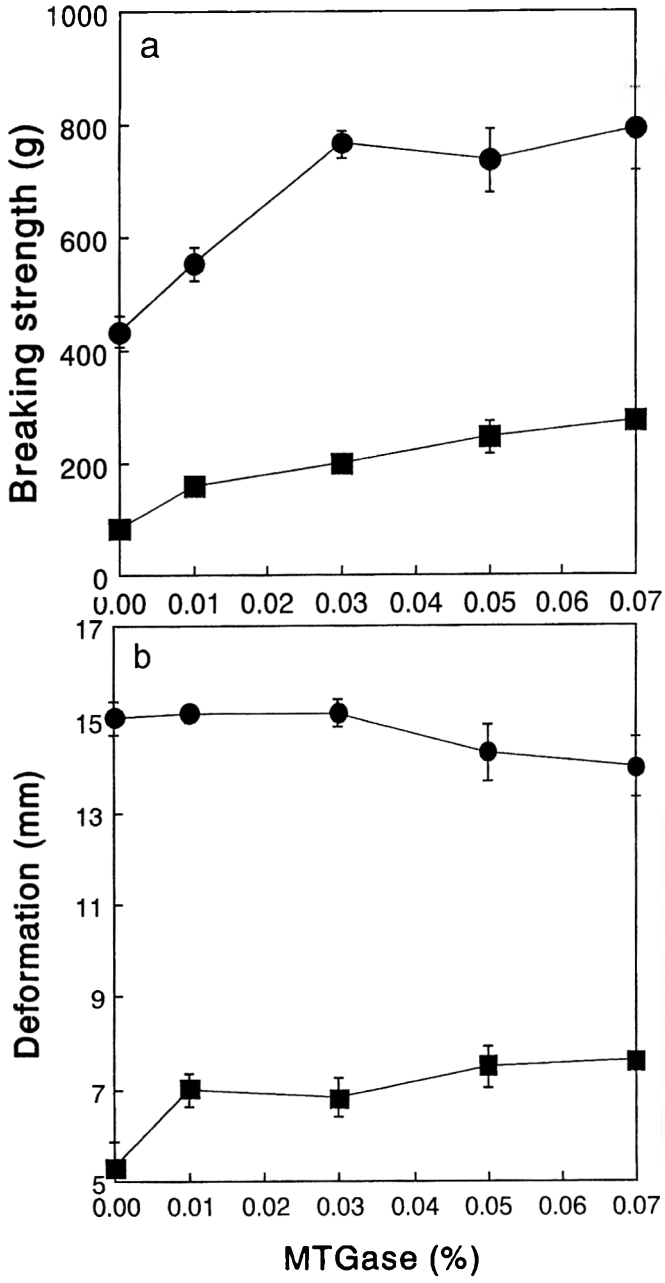

Fig. 3—Changes in breaking strength and deformation of kamaboko gels set 45°C for 30 min as related to MTGase concentration. H/A gel, ●; H/2 gel, ■. Vertical bars represent Standard deviation. For details, see Materials & Methods.

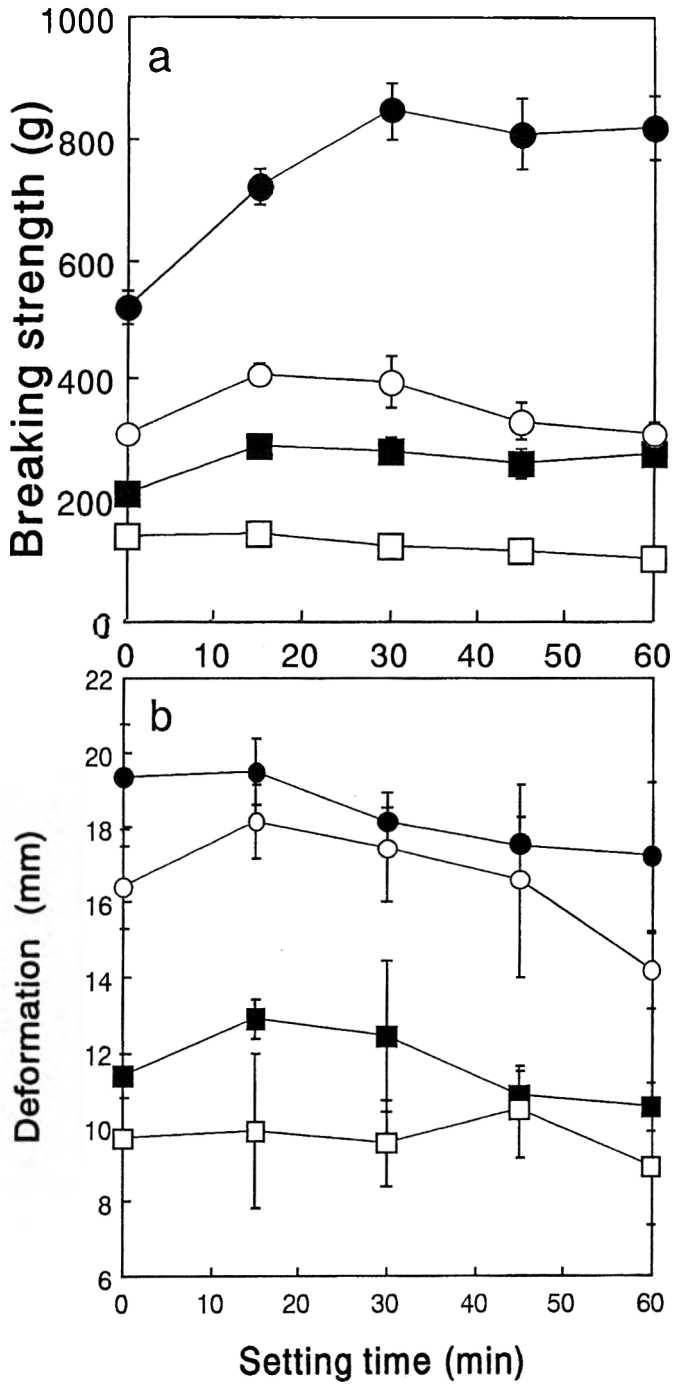

Fig. 4—Changes in breaking strength and deformation of kamaboko gels set at 45°C as related to setting time. H/A gel, ○ ●; H/2 gel, □ ■. Gels with MTGase added, filled symbols; gels without MTGase, open symbols. Vertical bars represent Standard deviation. For details, see Materials & Methods.

RESULTS

Low-temperature setting

Effect of the MTGase addition on kamaboko gels preincubated ("set") at 10°C for 16 hr was investigated (Fig. 1). The breaking strength in L/A gel without MTGase was as high as 800g and it slightly increased at 0.01% MTGase. But its overall increase was not much even at 0.07% MTGase. In L/2 gel, it increased gradually and depended on MTGase concentration (Fig. 1a). The deformation in L/A gel steadily decreased from a maximum at 0% MTGase, while it increased in L/2 gel as MTGase concentration increased (Fig. 1b). These results indicate that by addition of MTGase SA™ grade surimi sol resulted in gels that were more brittle, while addition of MTGase 2nd™ grade surimi sol resulted in gels with higher elasticity. Thus the MTGase addition increased elasticity more effectively in 2nd™ grade surimi than in SA™ grade surimi. And the effective amount of MTGase in L/2 gel was estimated to be around 0.03% MTGase (since the deformation reached maximum).

The effect of a 0.03% MTGase addition on kamaboko gels set at 10°C over setting time was also investigated (Fig. 2). The effect on LA gel was observed within 8 hr preincubation, although it was not notable. The breaking strengths of the gel with MTGase and the control were almost the same after 32 hr setting. In L/2 gel preincubated with 0.03% MTGase, the increment in the breaking strength was apparently larger than that of the control (Fig. 2a). The deformation retained up to 8 hr in L/A gel with MTGase; however, after 24 and 32 hr it decreased. Although the gels behaved similarly, the deformation in the MTGase sample was always lower than in the control at all

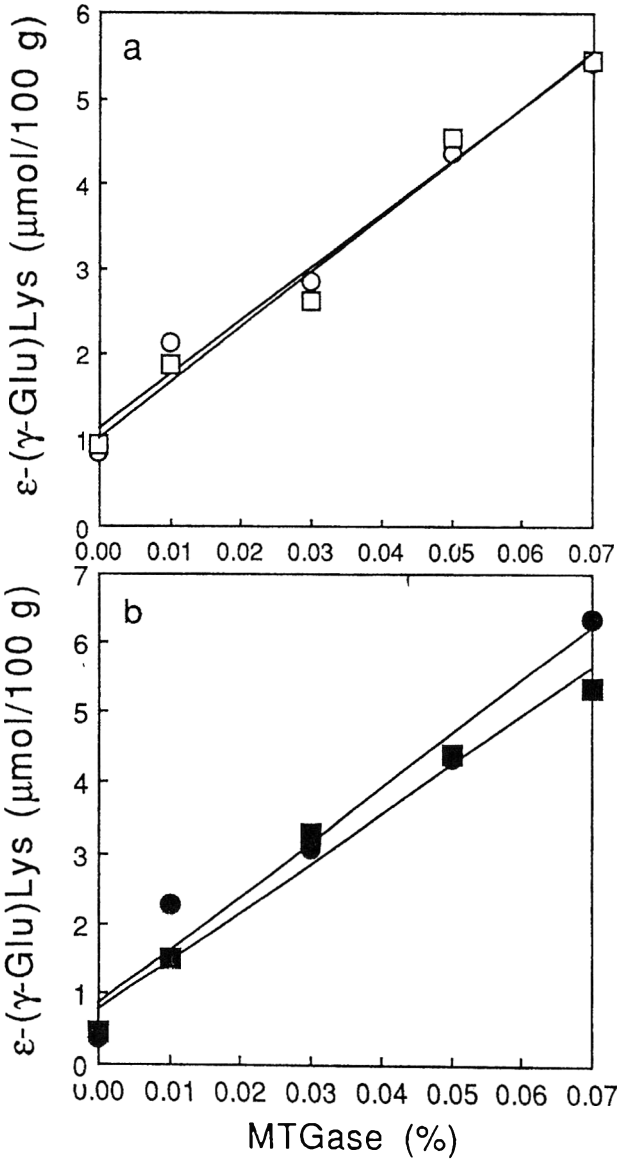

Fig. 5—Changes in ε-(γ-glutamyl)lysine content in kamaboko gels as related to MTGase concentration. (a) 10°C for 16-hr setting. L/A gel, ○; L/2 gel, □. (b) 45°C for 30-min setting conditions. H/A gel, ●; H/2 gel, ■. Each value is an average of two measurements on each corresponding gel.

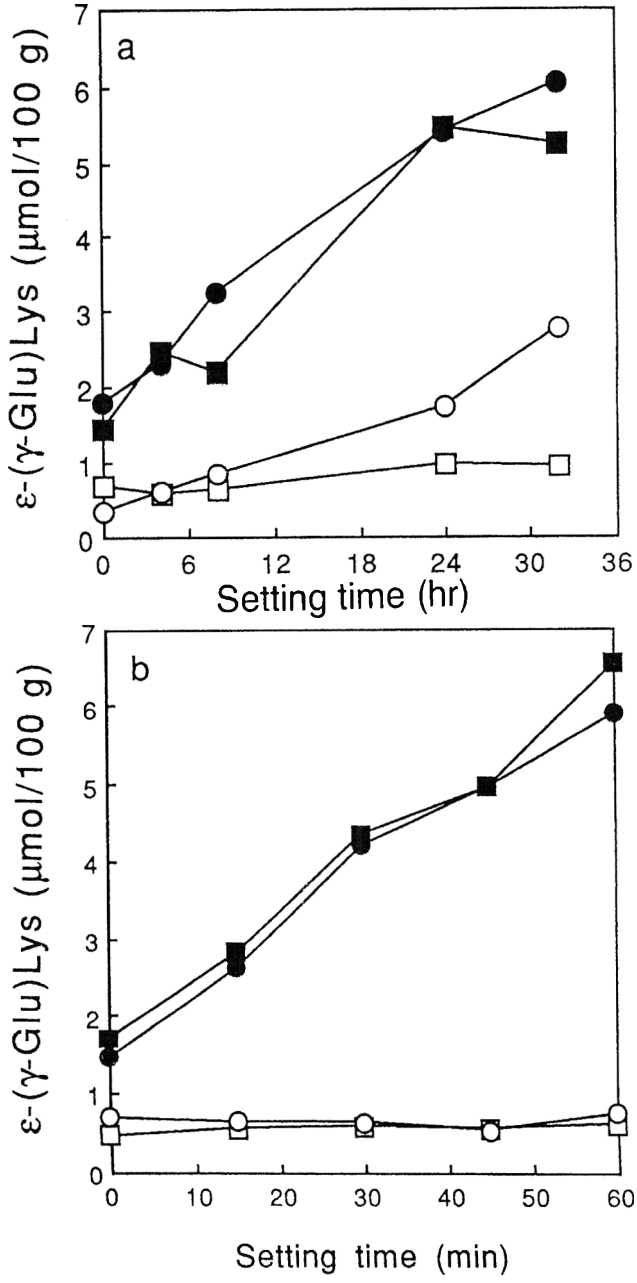

Fig. 6—Changes in ε-(γ-glutamyl)lysine content in kamaboko gels with 0.03% MTGase as related to setting time. (a) 10°C setting. L/A gel, ○ ●; L/2 gel, □ ■. (b) 45°C setting conditions. H/A gel, ○ ●; H/2 gel, □ ■. Gels with MTGase added, filled symbols; gels without MTGase, open symbols. Each value is an average of two measurements on each corresponding gel.

MTGase concentrations. In L/2 gels, the deformation fluctuated very little and values in MTGase-added gels were higher than in controls (Fig. 2b). Again by addition of MTGase in SA™ grade surimi paste, kamaboko gels became more brittle, although they gained in firmness; and in 2nd™ grade surimi paste the gels became more elastic. However, it may still be possible to form gels with high elasticity from SA™ grade surimi, if the MTGase reaction is terminated within 8 hr.

High-temperature setting

The effect of MTGase addition on kamaboko gels set at 45°C was also investigated (Fig. 3). The breaking strength in H/A gel, which was half that in gels set at 10°C (Fig. 1a), increased linearly up to 0.03% MTGase and afterward reached a plateau. In H/2 gel, it increased gradually as concentration increased (Fig. 3a). The deformation in H/A gel retained up to 0.03% MTGase, and then began to decrease. In H/2 gel, it increased rather sharply at 0.01% MTGase and plateaued afterwards (Fig. 3b). These results indicate that addition of up to 0.03% MTGase in SA™ grade surimi resulted in more viscoelastic gels. Excess amounts of MTGase impaired such effects, since the decrease in deformation indicated that the gels became rigid and brittle. Gels from 2nd™ grade surimi pastes showed increases in both breaking strength and deformation, indicating MTGase addition in 2nd™ grade surimi in 45°C setting contributed to elasticity. But the increment of the breaking strength in H/2 gel was slight, even at 0.07% MTGase, and less when compared with results in L/2 gel set at 10°C (Fig. 1a).

The effects of a 0.03% MTGase addition on kamaboko gels set at 45°C over setting time was investigated (Fig. 4). In H/A gel, the effect was notable when compared with the control. In H/2 gel the breaking strength of gels with MTGase was larger; however, the increment was small, even at 60 min (Fig. 4a). The deformation of H/A gel was retained up to 15 min, and started to decrease afterwards. The values, however, were always

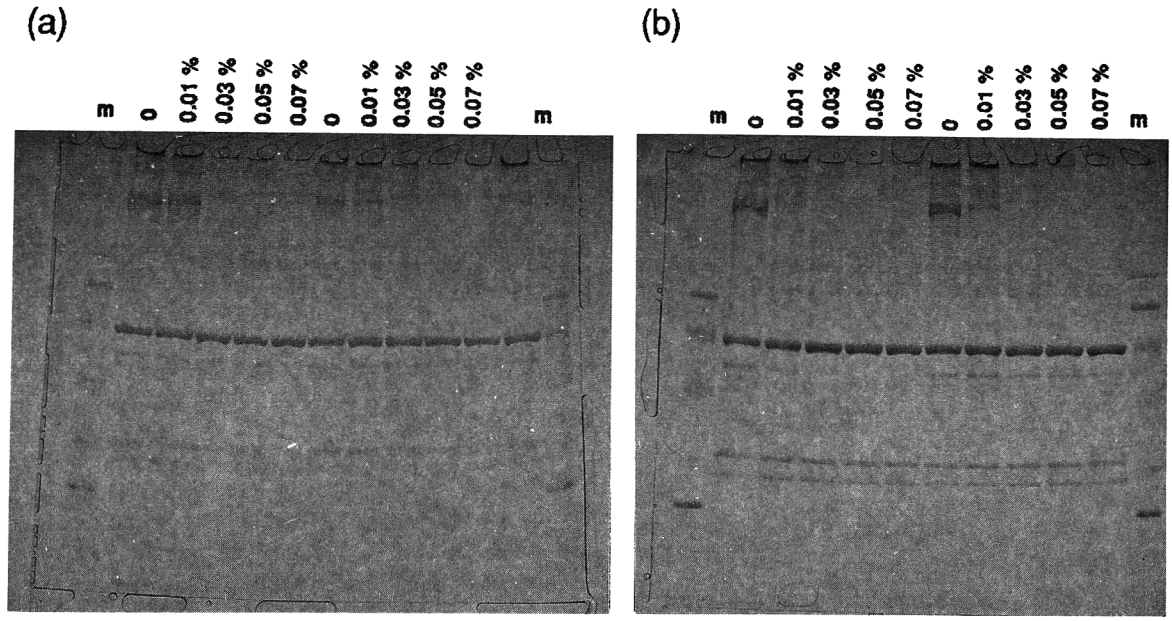

Fig. 7—Changes in SDS-PAGE pattern of myofibrillar proteins in kamaboko gels as related to MTGase concentration. Protein portions of each gel were dissolved in 8 M urea, 2% SDS, 2% β-mercaptoethanol, 20 mM Tris-HCl (pH 8.0) and applied (10 μg protein/lane). Numbers indicate enzyme concentration (w/w) "m" molecular weight marker (low-molecular-weight type, Pharmacia-LKB). (a) L/A and L/2 gels (or SA and 2nd grade surimi gels set at 10°C). (b) H/A and H/2 gels (or SA and 2nd grade surimi gels set at 45°C).

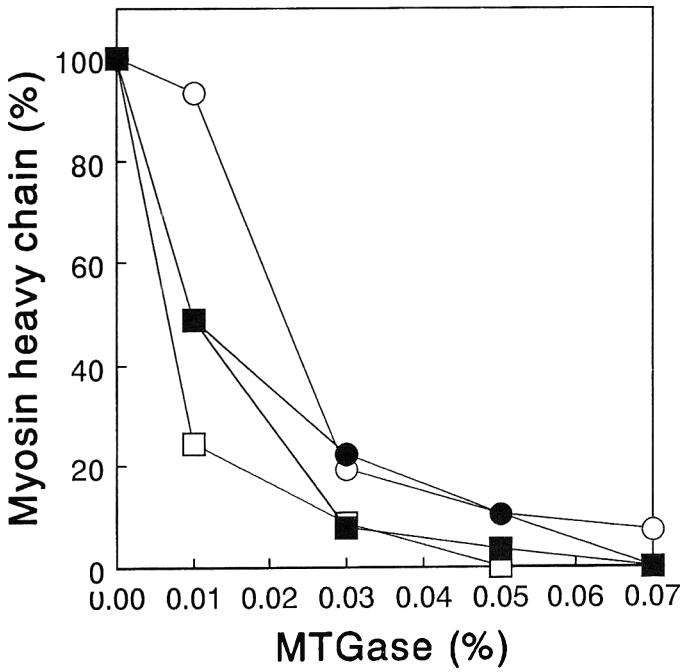

Fig. 8—Changes in myosin heavy chain content in kamaboko gels as related to MTGase concentration. L/A gel, ○; L/2 gel, □. H/A gel, ●; H/2 gel, ■. Myosin heavy chain contents in SDS-PAGE gels (Fig. 7) were estimated by densitometric analysis. For details, see Materials & Methods.

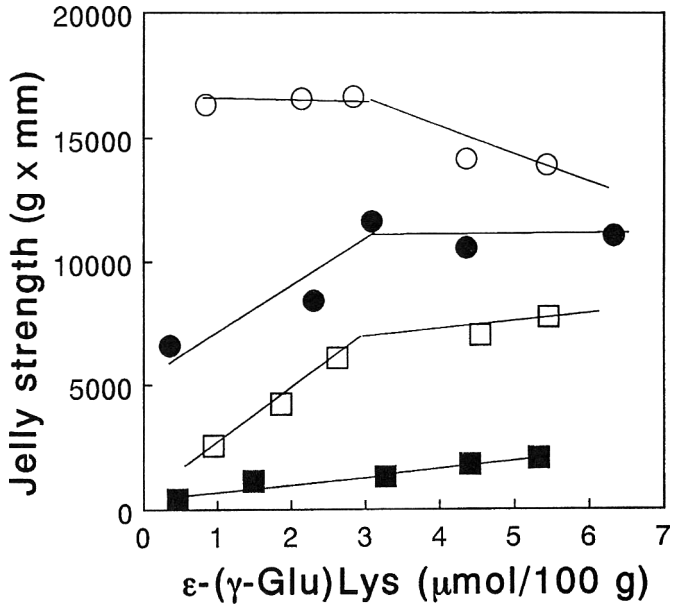

Fig. 9—Changes in jelly strength in kamaboko gels as related to ε-(γ-glutamyl) lysine content. L/A gel ○; L/2 gel □; H/A gel, ●; H/2 gel ■. ε-(γ-Glutamyl)lysine content was the same as shown in Fig. 5.

higher than controls. In H/2 gel, the deformation reached a maximum at 15 min and started to decrease beyond 15 min. Values were always higher than controls (Fig. 4b). These results indicated that with 0.03% MTGase, SA™ grade surimi paste remained elastic up until 15 min setting, and 2nd™ grade surimi paste also gained in elasticity around 15 min. However, the gain in breaking strength in 2nd™ grade surimi gel was negligible.

Determination of ε-(γ-Glu)Lys in gels

Formation of ε-(γ-Glu)Lys crosslink in all gels was investigated (Fig. 5 and 6). When SA™ and 2nd™ grade surimi pastes were set at 10°C for 16 hr, proportional increases in the ε-(γ-Glu)Lys content to MTGase concentration were observed, regardless of grade of surimi (Fig. 5a). Similarly, when SA™ and 2nd™ surimi pastes were set at 45°C for 30 min, the amounts of ε-(γ-Glu)Lys increased proportionally to enzyme concentration, regardless of grade (Fig. 5b). Amounts finally reached 5.5 and 6 μmol/100 g gel for 10°C and 45°C settings, respectively, at 0.07% MTGase and final amounts were almost the same. The proportionality in the two setting systems suggested that the catalytic action of MTGase and ε-(γ-Glu)Lys was generated enzymatically during setting. The difference in enzymatic reactivity was one-sixth between 10 and 40°C (data not shown); however, it was overcome by the duration of the reaction, for the setting time at 10°C was 16 hr. When SA™ and 2nd™ grade

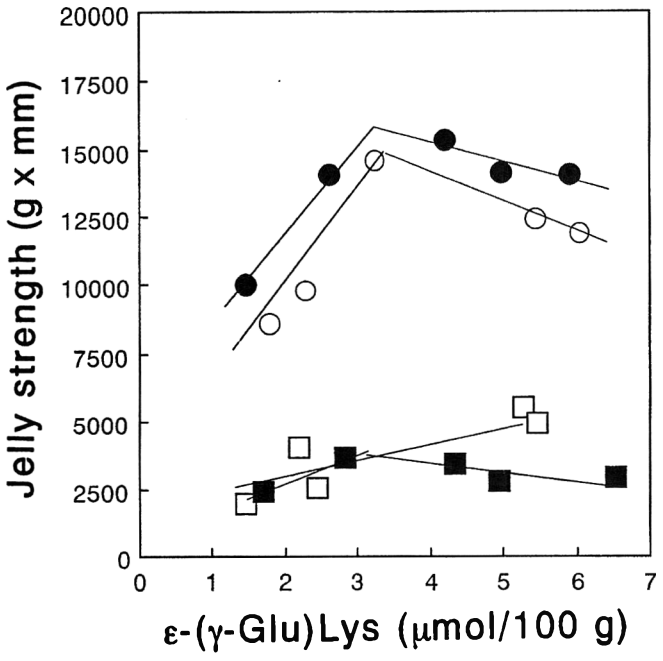

Fig. 10—Changes in jelly strength in kamaboko gels as related to ε-(γ-glutamyl) lysine content. 10°C and 45°C settings with 0.03% MTGase added. L/A gel ○; L/2 gel □; H/A gel ●; H/2 gel, ■. ε-(γ-Glutamyl)lysine content was the same as shown in Fig. 6.

surimis with 0.03% MTGase were set at 10°C, increases in ε-(γ-Glu)Lys content were observed and they were proportional to setting time, regardless of grade (Fig. 6a). When SA™ and 2nd™ surimis with 0.03% MTGase were set at 45°C, a similar proportionality in the ε-(γ-Glu)Lys content was observed, regardless of grade (Fig. 6b). The proportionality in the two setting systems suggested the catalytic action of MTGase and ε-(γ-Glu)Lys was generated enzymatically during setting. The amounts at 32 hr for 10°C setting and 60 min for 45°C setting were both around 6 μmol/100 g gel. Since setting times at 10°C were 32 hr, the difference in enzymatic reactivity was overcome by the duration.

Decreases in myosin heavy chain in gels

Changes in SDS-PAGE pattern of myofibrillar proteins over MTGase concentrations in both L and H gels were compared (Fig. 7). After densitometric quantitative measurement, the MHC contents were expressed relative to intensity of MHC of surimi gels prepared without MTGase (Fig. 8). When SA™ and 2nd™ surimi pastes were set at either 10°C for 16 hr or 45°C for 30 min, the amounts of MHC apparently decreased with increases in MTGase concentration, regardless of grade and setting conditions, and finally it reached 0%. Since no smaller-molecular-size peptides appeared in the SDS-PAGE gels, and the gels were treated with a buffer containing SDS, urea and mercaptoethanol, such a decrease in MHC may be due to crosslinking of MHC with ε-(γ-Glu)Lys, and not disulfide bonds.

Relationship between the ε-(γ-Glu)Lys crosslink content and gel properties

The relationship between the ε-(γ-Glu)Lys content and jelly strength was studied (Figs. 9 and 10). Up to 3 μmol/100 g ε-(γ-Glu)Lys the content correlated with jelly strength in H/A and L/2 gels (Fig. 9) with correlation coefficients of 0.85 and 0.99, respectively. In L/A gel, no change in jelly strength occurred up to 3 μmol/100 g ε-(γ-Glu)Lys. However, excess in the ε-(γ-Glu)Lys crosslink content started to lower jelly strength. In H/2 gel, the jelly strength steadily increased as ε-(γ-Glu)Lys content increased, but its increments were not large, as compared with the other three. These results indicated that the ε-(γ-Glu)Lys crosslink up to 3 μmol/100 g was effective in increasing in jelly strength. That is, the amount of MTGase, which produced 3 μmol/100 g ε-(γ-Glu)Lys, was 0.03% and that was enough to improve surimi gel properties. Similar relations were observed between the ε-(γ-Glu)Lys content and jelly strength (Fig. 10) in gels treated with 0.03% MTGase. Such relations were significant in SA™ grade surimi gels set at 45°C. And in 2nd™ grade surimi gels, a steady increase in jelly strength appears to indicate the effect of MTGase to increase elasticity in gels.

DISCUSSION

SEKI ET AL. (1990) and Araki and Seki (1993) reported the presence of TGase activity in fish, including Alaska pollock. Kimura et al. (1991) and Sato et al. (1992) demonstrated that formation of ε-(γ-Glu)Lys crosslinks occurred during the setting of surimi produced from flesh of hoki (*Macruronus novaezelandiae*) and sardine, respectively. The viscoelastic property of kamaboko gel has been thought to be influenced by several bonds, including ε-(γ-Glu)Lys crosslinks (Niwa, 1992). Formation of ε-(γ-Glu)Lys crosslinks by intrinsic TGases in fish products was reported (Sato et al., 1992; Kumazawa et al., 1993a). Tsukamasa et al. (1993) revealed the relationship between formation of ε-(γ-Glu)Lys crosslinks and gel-strength of sardine gels in low-temperature setting. Such intrinsic TGase activities, however, were smaller in 2nd™ grade than SA™ grade surimi (Kumazawa et al., 1993b).

Based on the evidence that MHC disappeared (Fig. 7 and 8) in the SDS-PAGE gels, we could assume that the catalytic action of MTGase occurred in the surimi paste during setting. Further, the presence of ε-(γ-Glu)Lys in the SA™ grade surimi gels prepared without MTGase (0 U/g) at 10°C suggested the presence of intrinsic TGases in the surimi (Fig. 5) and supported the previous finding by Seki et al. (1990), Kamath et al. (1992), and Araki and Seki (1993). An increase (2.5 μmol/100 g gel) in the ε-(γ-Glu)Lys content in SA™ gel set at 10°C (L/A gel in Fig. 6) supported the hypothesis that at high-temperature settings, such as 40 to 50°C, crosslinking due to the intrinsic TGases in surimi was suppressed (Kamath et al., 1992). The ε-(γ-Glu)Lys crosslink content finally reached 6 μmol/100 g gel in both 10 and 45°C-settings (Fig. 6). However, at 10°C setting, both the intrinsic TGases and MTGase were considered to exert their activity. Thus the increase due to MTGase in L/A set for 32 hr was estimated to be 3.5 μmol/100 g gel. Although MTGase would have a low activity (one-sixth of optimum value) at low temperatures, the setting time (32 hr) was long enough to produce such amount. At 45°C setting the increment (6 μmol/100g gel) in the ε-(γ-Glu)Lys crosslink was considered to be due to MTGase only.

The ε-(γ-Glu)Lys crosslink content up to 3 μmol/100 g gel was critical in gel properties, since beyond that point the increase in jelly strength slowed or reversed (Fig. 9 and 10). Therefore, formation of up to about 3 μmol of ε-(γ-Glu)Lys crosslink/100 g gel was considered effective in improving gel properties. This corresponded to ≈0.03% addition of MTGase (Fig. 5) or the 20 min and 45°C setting and 8 hr and 10°C setting (Fig. 6). The jelly strength of the gels set at 10°C was always higher than those set at 45°C (Fig. 9), though the amounts of ε-(γ-Glu)Lys crosslinks were almost the same. This may be because the jelly strength is the product of breaking strength and deformation and both parameters in the gels set at 10°C were higher than those set at 45°C (Figs. 1 and 3). The enhancement in hydrogen bonding at low temperature has been known (Cheftel et al. 1985), therefore, breaking strength may be fortified through enhancement of hydrogen bonds in the gels set at lower temperatures. Softening ("modori") of kamaboko gels is well known to occur during heating (Shimizu, 1987; Niwa, 1992). In our experiments, however, the setting temperature and period

were 45°C and 60 min, respectively, and in those conditions, no changes in jelly strength and breaking strength in Alaska pollock surimi gel have been reported (Numakura et al., 1990). Thus the results indicate that under low temperature, as well as high temperature, conditions, MTGase could increase the jelly strength and the impairment in gel properties would be due to excess formation of the ε-(γ-Glu)Lys crosslink. Further, such an improving effect seemed to be greater at low temperatures than at high temperatures. The setting periods were usually longer to activate intrinsic TGases. Also, note that our gels were cooked at 85°C for 30 min, as commercial products are. Thus the breaking strength of the gels could be influenced by not only ε-(γ-Glu)Lys, but also many other factors, such as unfolding of protein molecules by heat, disulfide bonds, hydrophobic interactions, hydrogen bonding and proteolytic degradation. These are all involved in the expression of viscoelasticity.

In conclusion, our study revealed that addition of up to 0.03% (w/w) MTGase in surimi sols was adequate to improve jelly strength or gel properties of kamaboko gels. Its influence on other factors affecting gel properties might be obscured through formation of up to 3 μmol of the ε-(γ-Glu)Lys crosslink in a 100-g gel. Also, the effect of MTGase to improve the quality of gels was obvious for the manufacture of gels from 2nd™ grade surimi in low-temperature and from SA™ grade surimi in high-temperature settings.

REFERENCES

Ando, H., Adachi, M., Umeda, K., Matsuura, A., Nonaka, M., Uchio, R., Tanaka, H., and Motoki, M. 1989. Purification and characteristics of a novel transglutaminase derived from microorganisms. Agric. Biol. Chem. 53: 2613–2617.

Araki, H. and Seki, N. 1993. Comparison of reactivity of transglutaminase to various fish actomyosins. Nippon Suisan Gakkaishi 59: 711–719.

Cheftel, J.C., Cuq, J.-L., and Lorient, D. 1985. Amino acids, peptides, and proteins. Ch. 5 in *Food Chemistry*, O.R. Fennema (Ed.), p. 246–369. Marcel Dekker, Inc., New York.

Folk, J.E. 1980. Transglutaminases. Ann. Rev. Biochem. 49: 517–531.

Folk, J.E. and Chung, S.I. 1973. Molecular and catalytic properties of transglutaminases. Adv. Enzymol. 38: 109–191.

Folk, J.E. and Cole, P.W. 1966. Mechanism of action of guinea pig liver transglutaminase. J. Biol. Chem. 241: 5518–5525.

Folk, J.E. and Finlayson, J.S. 1977. The ε-(γ-glutamyl)lysine cross-link and the catalytic role of transglutaminase. Adv. Protein Chem. 31: 1–133.

Hamann, D.D., 1992. Viscoelastic properties of surimi seafood products. Ch. 5 in *Viscoelastic Properties of Foods*, M.A. Rao and J.F. Steffe (Ed.), p. 157–171. Elsevier Applied Science, London and New York.

Icekson, I. and Apelbaum, A. 1987. Evidence for transglutaminase activity in plant tissue. Plant Physiol. 84: 972–974.

Kamath, G.G., Lanier, T.C., Foegeding, E.A., and Hamann, D.D. 1992. Non-disulfide covalent cross-linking of myosin heavy chain in "setting" of Alaska pollock and Atlantic croaker surimi. J. Food Biochem. 16: 151–172.

Kimura, I., Sugimoto, M., Toyoda, K., Seki, N., Arai, K., and Fujita, T. 1991. A study on the cross-linking reaction of myosin in kamaboko "suwari" gels. Nippon Suisan Gakkaishi 57: 1389–1396.

Kishi, H., Nozawa, H., and Seki, N. 1991. Reactivity of muscle transglutaminase on carp myofibrils and myosin B. Nippon Suisan Gakkaishi 57: 1203–1210.

Klein, J.D., Guzman, E., and Kuehn, G.D., 1992. Purification and partial characterization of transglutaminase from *Physarum pholycephalum*. J. Bacteriol. 174: 2599–2605.

Kumazawa, Y., Seguro, K., Takamura, M., and Motoki, M. 1993a. Formation of ε-(γ-glutamyl) lysine cross-link in cured horse mackerel meat induced by drying. J. Food Sci. 58: 1086–1089.

Kumazawa, Y., Numazawa, T., Seguro, K., Takamura, M., and Motoki, M. 1993b. Participation of transglutaminase in the manufacturing process of "kamaboko." Paper no. 124A, presented at 53rd Annual Meeting of the Institute of Food Technologists, Chicago, IL, July 10–14.

Kurth, L. and Rogers, P.J. 1984. Transglutaminase-catalyzed cross-linking of myosin to soya protein, casein and gluten. J. Food Sci. 49: 573–589.

Laemmli, U.K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227: 680–685.

Lanier, T.C., Manning, P.K., Zetterling, T., and MacDonald, G.A. 1992. Process innovations in surimi manufacture. Ch. 7 in *Surimi Technology*, T.C. Lanier and C.M. Lee (Ed.), p. 167–179. Marcel Dekker, Inc., New York.

Margosiak, S.A., Alavarets, M.E., Louie, D., and Kuhen, G.D. 1990. Identification of the large subunit of ribulose 1,5-bisphosphate carboxylase/oxygenase as a substrate for transglutaminase in *Medicago sativa* L. (alfalfa). Plant Physiol. 92: 88–96.

Niwa, E. 1992. Chemistry of surimi gelation. Ch. 16 in *Surimi Technology*, T.C. Lanier and C.M. Lee (Ed.), p. 167–179. Marcel Dekker, Inc., New York.

Nonaka, M., Sakamoto H., Toiguchi, S., Kawajiri, H., Soeda, T., and Motoki, M., 1992. Sodium caseinate and skim milk gels formed by incubation with microbial transglutaminase. J. Food Sci. 57: 1214–1218, 1241.

Nonaka, M., Tanaka, H., Okiyama, A., Motoki, M., Ando, H., Umeda, K., and Matsuura, A. 1989. Polymerization of several proteins by Ca^{2+}-independent transglutaminase derived from microorganisms. Agric. Biol. Chem. 53: 2619–2623.

Numakura, T., Kimura, I., Toyoda, K., and Fujita, T. 1990. Temperature-dependent changes in gel strength and myosin heavy chain of salt-ground meat from walleye pollack during setting. Nippon Suisan Gakkaishi 56: 2035–2043.

Numakura, T., Seki, N., Kumura, I., Toyoda, K., Fujita, T., Takama, K., and Arai, K., 1985. Cross-linking reaction of myosin in the fish paste during setting (suwari). Bull. Jap. Soc. Sci. Fish 51: 1559–1565.

Ramanujam, M.V. and Hageman, J.H. 1990. Intracellular transglutaminase (EC 2.3.2.13) in a procaryote: Evidence from vegetable and sporulating cells of *Bacillus subtilis* 168. FASEB J. 4: A3630.

Sato, K., Tsukamasa, Y., Imai, C., Ohtsuki, K., Shimizu, Y., and Kawabata, M. 1992. Improved method for identification and determination of e-(γ-glutamyl)lysine cross-link in protein using proteolytic digestion and derivatization with phenyl isothiocyanate followed by high-performance liquid chromatography separation. J. Agric. Food Chem. 40: 806–810.

Seki, N., Uno, H., Lee, N.-H., Kimura, I., Toyoda, K., Fujita, T., and Arai, K. 1990. Transglutaminase activity in Alaska pollack muscle and surimi, and its reaction with myosin B. Nippon Suisan Gakkaishi 56: 125–132.

Shimizu, K., 1981. Kamaboko no Ashi (ash of kamaboko). In *Shokuhin no Bussei (Physiological Properties of Foods)*, Y. Yamano (Ed.), p. 149–160. Shokuhin Shizai Kenkyukai Inc. Co., Tokyo.

Shimizu, K., 1987. Gel Keiseinou (gel-forming ability). Ch. 3 in *Gyoniku Neri Seihin (Fish Paste Products)*, M. Okada, T. Imaki, and M. Yokonobu (Ed.), p. 42–66. Kouseisha Kouseikaku, Tokyo.

Signorini, M., Beninati, S., and Bergamini, C.M. 1991. Identification of transglutaminase activity in the leaves of silver beet (*Beta vulgaris* L.) J. Plant Physiol. 137: 547–552.

Tanaka, H., Nonaka, M., and Motoki, M. 1990. Polymerization and gelation of carp myosin by microbial transglutaminase. Nippon Suisan Gakkaishi 56: 1341.

Tsukamasa, Y. and Shimizu, Y. 1991. Factors affecting the transglutaminase-associated setting phenomenon in fish meat sol. Nippon Suisan Gakkaishi 57: 535–540.

Tsukamasa, Y., Sato, K., Imai, Shimizu, Y., Imai, C., Sugiyama, M., Minegishi, Y., and Kawabata, M. 1993. ε-(γ-Glutamyl)lysine crosslink formation in sardine myofibril sol during setting at 25°C. J. Food Sci. 58: 785–787.

Urch, M.J., 1988. Fish and fish products. In *Food Industries Manual*, Ch. 2, M.D. Ranken (Ed.), p. 33–69. Blackie, Glasgow and London.

Wang, J., Kimura, I., Satake, M., and Seki, N. 1994. Effect of calcium ion concentration on the gelling properties and transglutaminase activity of walleye pollack surimi paste. Fisheries Science 60: 107–113.

Washizu, K., Ando, K., Koikeda, S., Hirose, S., Matsuura, A., Takagi, H., Motoki, M., and Takeuchi, K. 1994. Molecular cloning of the gene for microbial transglutaminase from *Streptoverticillium* and its expression in *Streptomyces lividans*. Biosci. Biotech. Biochem. 58: 82–87.

Wicker, L., Lanier, T.C., Knopp, J.A., and Hamann, D.D. 1989. Influence of various salts of heat-induced ANS fluorescence and gel rigidity development of tilapia (*Serotherodon aureus*) myosin. J. Agric. Food Chem. 37: 18–22.

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Fermentation of Cucumbers Without Sodium Chloride

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ABSTRACT

Cucumbers were successfully fermented and stored in the absence of sodium chloride (salt) under laboratory conditions, provided the fruit were blanched (3 min, 77°C) before brining in a calcium acetate buffer and the brine inoculated with *Lactobacillus plantarum*. Bloater formation was prevented by blanching even when brines were not purged of CO_2. Firmness of cucumbers was similar in salt-free brines or those containing salt after 1 mo, but firmness of salt-free cucumbers was lower after storage for 12 mos. Under pilot-scale, commercial conditions, however, the cucumbers were severely bloated, and the firmness was unacceptable after storage for 7 mo, due apparently to microbial recontamination after blanching.

Key Words: cucumbers, fermentation, sodium chloride, *Lactobacillus plantarum*, blanching

INTRODUCTION

THE U.S. ENVIRONMENTAL PROTECTION AGENCY has proposed a limit of 230 ppm of chloride in freshwater bodies (Fed. Reg., 1987). Many pickle companies throughout the U.S. have difficulty meeting the 230 ppm limit in discharges from their plant operations, mainly because about 40% of the pickling cucumber crop is temporarily preserved in large vessels containing sodium chloride brine. When needed for processing into finished products, salt is leached from the brined cucumbers and, after biodegradation of organic residues, is discharged directly into streams or into municipal waste systems. The industry has made efforts to reduce chloride wastes. They have reduced concentrations of salt used to store brined cucumbers and are replacing wooden tanks which tend to leak with nonleaking polyethylene or fiberglass tanks.

The addition of $CaCl_2$ or calcium acetate to fermentation brines has reduced the concentration of sodium chloride necessary for retaining textural properties of brined cucumbers (Buescher et al., 1979; 1981; Fleming et al., 1978; 1987; McFeeters and Fleming, 1991). However, microbial instability of fermented cucumbers is a problem when the salt concentration is too low. Cucumbers brined at 2.3% NaCl underwent a normal lactic acid fermentation, resulting in >1% lactic acid and pH 3.7 (Fleming et al., 1989). Subsequently, however, the cucumbers spoiled due to production of butyric acid and other products formed by undesirable bacteria while the level of lactic acid was reduced.

Our objectives were to determine the feasibility of fermenting and storing cucumbers in the absence of NaCl by blanching the cucumbers before brining. This method was compared to a procedure developed for fermentation and storage in anaerobic tanks at relatively low NaCl concentration (Fleming et al., 1988). Also, the use of a malate-negative (unable to produce CO_2 from malic acid) culture of *Lactobacillus plantarum* to ferment the cucumbers was evaluated. Predominant growth by such a culture could eliminate the need for purging to prevent bloater formation.

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MATERIALS & METHODS

Cucumber brining

Fresh pickling cucumbers, size 2B (3.5–3.8 cm diameter) or 3A (3.8–4.4 cm) were washed in either a reel washer (laboratory experiments) or brush washer (pilot tank experiment). They were fermented by two different treatments, blanched, no salt (BNS), and salt, not blanched (SNB), (Fig. 1). Cucumber blanching was done in a water-jacketed steam kettle for laboratory experiments and in a heated water flume for the pilot experiment. Unless otherwise specified, blanching was 3 min at 77°C. The cucumbers were packed into 3.8 L jars with expansion reservoirs (Fleming et al., 1973), 19 L pails, or 4,428 L fiberglass tanks to occupy 55 to 60% of the container volume. All laboratory fermentations were duplicated, and data reported are averages of duplicates. Pilot-scale fermentations were not duplicated. The pilot tanks and related cucumber handling methods were as reported (Fleming et al., 1983). The cover brines consisted of either calcium acetate buffer (to equilibrate with cucumbers to 0.053 M acetic acid and 0.018 M calcium), as previously described (Fleming et al., 1988), and/or other components (noted in footnotes to tables). The nitrogen purging rate was 25 mL/min for jars and 400 mL/min for pilot tanks when purging was applied.

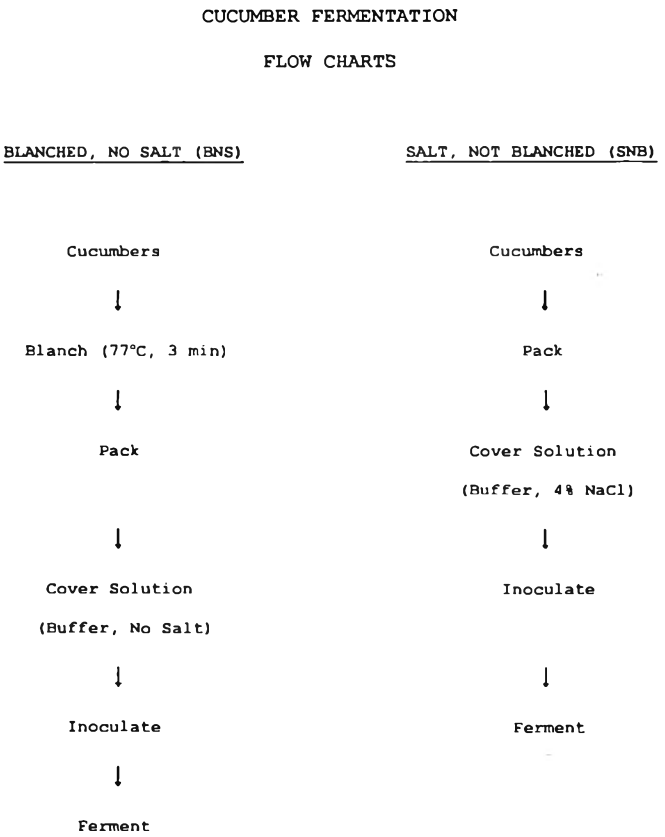

Fig. 1—Flow diagram for two procedures used for fermentation of cucumbers. The calcium acetate buffer (pH 4.7 ± 0.1) indicated in the cover solutions contained acetic acid and calcium hydroxide to attain concentrations, after equilibrium with cucumbers, of 53 and 18 mM, respectively.

Table 1—Effect of BNS and SNB treatments on cucumbers fermented by MDC⁻ and MDC⁺ cultures of *L. plantarum*[z]

Culture	Cucumber treatments		Final brine composition					Cucumber quality	
	Blanched	Salt	pH	Malic acid (mM)	Lactic acid (mM)	Residual sugar (mM)	CO_2 (mg/100 mL)	Bloater index	Firmness FPT (kg)
MDC⁻	−	+	3.5	0.3	120	4	78[c]	22[a]	9.8[a]
	+	−	3.6	4.9	109	1	62[d]	0[b]	9.6[a]
MDC⁻	−	−	3.7	1.2	124	1	114[a]	27[a]	9.5[a]
	+	+	3.5	6.2	103	11	48[ec]	1[b]	10.0[a]
MDC⁺	−	+	3.5	2.4	121	1	76[c]	22[a]	9.1[a]
	+	−	3.7	2.2	129	1	91[b]	4[b]	9.6[a]

[z] Size 3A cucumbers (208 1g) were blanched (3 min, 77°C) or unblanched and packed into 3.7L jars and covered with calcium acetate buffer with or without NaCl as noted. Analyses were made after incubation at 26°C for 1 month. Letters within columns designate statistically significa nt differences ($P \leq 0.05$). BNS = blanched, no salt; SNB = salt, not blanched.

Microbial cultures

Microbial cultures included *L. plantarum* WSO (MDC⁺) and a mutant, M6, from that culture that did not produce CO_2 from lactic acid (MDC⁻) (Daeschel et al., 1984; McDonald et al., 1993). The lactic acid bacteria (LAB) were introduced at a rate of 10^6/mL of brined cucumbers. Brines were inoculated after brine was added to the cucumbers in laboratory fermentations and before brine was added to cucumbers in pilot-scale fermentations. The *L. plantarum* (MDC⁻) culture was prepared as a frozen concentrate by Chr. Hansen's Laboratory (Milwaukee, WI) under a sub-license agreement for a patent (Daeschel et al., 1987). The other culture was grown overnight in cucumber juice broth containing 0.018 M calcium acetate and 2% NaCl (McDonald et al., 1993).

Microbial analyses

General procedures for enumeration of microorganisms were described by Fleming et al. (1992a). Media included standard methods agar (PCA, BBL Microbiology Systems, Cockeysville, MD) for aerobes, violet red bile agar (BBL) + 1% glucose (VRBG) for *Enterobacteriaceae*, MRS broth (Difco Laboratories, Detroit, MI) + 1.5% agar + 0.02% sodium azide (MMRS) for LAB, and standard methods agar (BBL) + 0.1 mg/mL of chlortetracycline HCl + 0.1 mg/mL of chloramphenicol for yeasts (YM). All pour plates were duplicated and incubated at 30°C. Microbial colonies in VRBG plates were enumerated after 24 hr, PCA and MMRS plates after 48 hr, and YM plates after 72 hr.

A total of 100 colonies was isolated from MMRS plates of each pilot fermentation at each sampling time for the purpose of determining percentages of LAB that were malate-negative. Isolated colonies were picked and inoculated into individual microtiter wells containing MD broth (Daeschel et al., 1984). Growth in this broth allows differentiation between MDC⁺ and MDC⁻ cultures and has previously been used to determine predominance of the MDC⁻ culture under laboratory conditions (McDonald et al., 1993).

Chemical analyses

Malic, lactic, acetic, propionic, and butyric acids and ethanol, mannitol, glucose, and fructose were measured in fermentation brines using high performance liquid chromatography (HPLC) (McFeeters et al., 1984). An Aminex HPX-87H column (Bio-Rad Laboratories, Richmond, CA) with a cation guard column was used for separation of acids, and an Aminex HPX-87C column (Bio-Rad) with cation and anion guard columns was used for separation of sugars. A refractive index detector was used for quantification of sugars, and a UV detector (210 nm) was used for quantification of acids. In order to detect malic acid using HPLC, reduction of fructose in the sample was necessary. The procedure of McFeeters et al. (1993) was used for fructose reduction. CO_2 was determined according to the method of Fleming et al. (1974). Glucose and fructose concentrations in raw cucumbers were determined by blending four lots of four cucumbers each and determining the average values for the 4 composite blendings.

Product evaluation

Bloater damage (expressed as bloater index, i.e., relative proportion of tissue damaged) and visual cure of the cucumbers were determined as reported (Fleming et al., 1977). Firmness of the cucumbers was determined with a USDA Fruit Pressure Tester (FPT) with a 0.79 cm tip and expressed as kg force (Bell et al., 1955). Since cucumbers were severely bloated in some treatments, firmness of all fruit was determined after they had been longitudinally sliced. Thus, the procedure was to slice the fruit first for bloater evaluation, and then to test firmness of 20 unbloated fruit halves. A comparison of firmness of whole vs half fruit (50 of each) was made on unbloated fruit from the middle section of the SNB pilot tank. The firmness means were 6.8 kg for half and 7.2 kg for whole fruit. This 0 4 kg difference in firmness was not significant ($P > 0.23$).

Statistical analyses

The General Linear Model Procedure of SAS (SAS Institute, Cary, NC) was used to compute all statistical inferences. The experimental design for the pilot tank experiment was a nonreplicated complete block, while the laboratory experiment was a duplicated complete block.

RESULTS

Laboratory fermentation of cucumbers

The effects of the BNS and SNB treatments (Fig. 1) of cucumbers on fermentation by MDC⁻ and MDC⁺ strains of LAB were tested under laboratory conditions. The cucumbers fermented normally by both cultures and under both brining treatments. However, cucumbers fermented faster in the absence of salt and when blanched, as was expected. The fermentations were completed after 1 mo, as evidenced by absence of sugars and cessation of acid production, at which time chemical compositions and product quality were determined (Table 1). The residual sugar in the brine had been reduced to 1–11 mM from a calculated initial concentration of 129 mM in the raw fruit.

The fermentation brines were not purged, thus the CO_2 concentration was permitted to increase to a maximum. In the SNB treatment, the CO_2 concentration by both the MDC⁻ and MDC⁺ strains was similar (76 vs 78 mg/100 mL, respectively). In the BNS treatment, however, the CO_2 concentration was notably higher in cucumbers fermented by the MDC⁺, as compared to the MDC⁻ strain (91 vs 62 mg/100 mL). The higher concentration of lactic acid with the MDC⁺ strain (129 mM) than with the MDC⁻ strain (109 mM) was consistent with expectation, since lactic acid is an end-product of malate decarboxylation. However, difference in CO_2 concentration could not be accounted for on the basis of malate decarboxylation since the difference in residual malate concentrations between MDC⁺ and MDC⁻ strains was only 2.7 mM for the BNS treatments (Table 1).

The highest concentration of CO_2 (114 mg/100 mL) was reached when the cucumbers were not heated and no salt was added (MDC⁻ control treatment). The lowest concentration of CO_2 (48 mg/100 mL) was reached when the cucumbers were blanched and salt was added (MDC⁻ control treatment). The MDC⁻ culture did not appear to predominate the SNB fermentations in our results. Breidt et al. (1992) showed predominance, as evidenced by high residual malate concentration and differential enumeration of MDC⁻ and MDC⁻ LAB. Differences in concentrations of natural MDC⁻ LAB in the two studies were probably responsible for these differences.

Bloater damage in fermented cucumbers was slight when cucumbers were fermented by BNS treatment, though they were fermented by the MDC⁺ culture, and the CO_2 concentration

Table 2—Chemical changes of cucumbers fermented by the BNS and SNB procedures by *L. plantarum* (MDC) in pilot tanks[a]

Fermentation time (days)	Blanched, no salt (BNS)					Salt, not blanched (SNB)				
	CO_2, (mg/100 mL)	Malic acid, (mM)	pH	Titratable acidity (%)	Sugar (%)	CO_2 (mg/100 mL)	Malic acid (mM)	pH	Titratable acidity, (%)	Sugar (%)
0	—	0.0	4.9	0.35	0.00	—	0.0	4.7	0.39	0.00
1	9	1.1	4.8	0.35	0.01	8	0.0	4.7	0.39	0.01
2	67	0.8	4.3	0.39	0.25	43	4.0	4.9	0.25	0.40
3	108	0.0	4.0	0.76	0.15	50	9.4	4.5	0.33	0.46
4	119	0.0	3.9	0.87	0.03	68	3.1	4.6	0.32	0.46
6	150	0.0	3.9	1.04	0.01	122	0.9	3.9	0.73	0.39
8	106	0.0	3.8	0.99	0.01	101	0.0	3.7	0.87	0.29
Purging started[b]										
10	41	0.0	3.8	0.98	0.01	58	0.0	3.7	0.88	0.24
16	34	0.0	3.7	1.06	0.01	30	0.0	3.6	1.01	0.09
30	12	0.0	3.7	1.01	—	10	0.0	3.5	1.07	—

[a] Size 2B cucumbers were fermented in 4,428L pilot tanks. Calcium acetate buffer with or without salt was added as cover liquor at 40% volume of the tank contents. Salt, when added, equalized at 4.4%. Blanching was for 3 min at 77°C.

[b] Purging with nitrogen was started after 8 days at a rate of 400 mL/min. The fermentations occurred at ambient temperature (about 26°C).

reached 91 mg/100 mL (Table 1). No bloater damage was evident in cucumbers fermented by the BNS treatment with the MDC^- culture. Bloater damage was severe in cucumbers fermented by SNB treatment, whether fermented by MDC^+ or MDC^- culture (Table 1).

Firmness of cucumbers from all treatments was excellent. There were no statistically significant differences among any of the treatments ($P \geq 0.05$). Since some of cucumbers were bloated, firmness determinations were made on cucumber halves from fruit that had not bloated.

The pH of brines from the various fermentations ranged from 3.5 to 3.7. There was no evidence of propionic and butyric acids or propanol. The products had desirable aromas, with no evidence of fermentation by undesirable microorganisms.

Pilot fermentation of cucumbers

Cucumbers were fermented in 4,428-L fiberglass tanks by the BNS and SNB treatments, using the *L. plantarum* MDC^- culture (Table 2). The CO_2 concentrations reached 106 and 101 mg/100 mL for the BNS and SNB treatments, respectively, after 8 days (Table 2). This was considerably higher than that reached in laboratory fermentations for the MDC^- culture after 1 mo (Table 1). Since purging of brines by nitrogen was not begun until after 8 days, the CO_2 concentration was an accumulation until that time, and indicated that microorganisms other than the *L. plantarum* MDC^- culture were active. After 30 days, no fermentable sugar remained in the brine, and the titratable acidity (calculated as lactic) had reached 1.01 and 1.07%, respectively, for the BNS and SNB treatments. The total fermentable sugars (glucose and fructose) in the raw cucumbers was 117 mM. The brine pH for the SNB treatment was 3.5 and for the BNS was 3.7. There was no evidence of butyric acid in brines of either treatment.

Microbial changes during fermentations were followed (Fig. 2). As in the laboratory study, the cucumbers fermented more rapidly in the BNS than in the SNB treatment. The numbers of LAB and total aerobes were nearly identical throughout the first 15 days of SNB fermentation. By comparison, total aerobes were slightly and consistently higher in numbers than LAB during the same period in the BNS fermentation (Fig. 2). We did not determine the reason for this difference, or if the bacteria that grew on the total aerobe plates were actually LAB that failed to grow on the MMRS medium, or were non-LAB. An initial 2-log cycle reduction occurred in *Enterobacteriaceae* count of the BNS, as compared to the SNB-treated cucumbers. Yeast counts were higher in the BNS than the SNB treatment, but never exceeded 1,000/mL and trended lower in both fermentations after about 5 days.

Quality of fermented cucumbers was evaluated after 1 and 7 mo storage in the pilot tanks (Table 3). For comparative purposes, cucumbers taken from the same lot fermented in the pilot tanks were also fermented in 19-L plastic pails under laboratory conditions. The bloater index was relatively high for the BNS treatment compared to the SNB treatment in pilot tanks (Table 3). The index was greater in the top than the middle section, consistent with previous results (Fleming et al., 1977). Also, visual cure was greater in the middle section, as reported previously. Cucumbers were firmer in the SNB than BNS treatment, and the firmness was maintained over a storage period of 7 mo. Firmness was significantly lower ($P \leq 0.05$) in the BNS than the SNB treatment 1 mo after brining, and after 7 mo the firmness had diminished to an unacceptable level.

In contrast, firmness of BNS and SNB cucumbers was similar when fermented under laboratory conditions (Table 3). However, there was a slight reduction in firmness of BNS cucumbers after storage for 12 mo, while there was a slight increase in firmness of SNB cucumbers after 12 mo. The BNS cucumbers had no bloater damage after 1 mo, while SNB cucumbers were slightly bloated.

DISCUSSION

RESULTS OF THIS STUDY indicate that successful fermentation of cucumbers in the absence of added sodium chloride may be possible. Under laboratory conditions, fermented cucumbers of good quality were obtained in the absence of added salt, provided the fruit were blanched prior to fermentation (BNS treatment). Under pilot-scale conditions, however, cucumbers fermented in the absence of salt were notably less firm after fermentation, and the quality deteriorated greatly during storage of 7 mo. Microbial recontamination after blanching was probably responsible for the lesser quality of cucumbers fermented by the BNS procedure under pilot-scale conditions. For the BNS procedure to be successfully applied on a commercial scale, more aseptic procedures may be required. The U.S. pickle industry currently is not structured to implement highly aseptic procedures in the handling of fresh cucumbers for brine-stock storage.

The BNS procedure provides the potential advantage of eliminating the need for purging to prevent bloater damage. Cucumbers blanched before fermentation by the MDC^- *L. plantarum* culture did not bloat under laboratory conditions, whether salt was present or absent. Heating may have inactivated microorganisms or enzymatic activity of the cucumber tissue itself. Further studies are needed to establish minimum heating requirements to prevent bloater formation without purging. It is likely that microbial inactivation is important since heated cucumbers bloated under pilot conditions where conditions were less aseptic.

An obvious advantage of BNS treatment was the removal of indigenous microorganisms and the possibility of controlled fermentation by added microorganisms with desirable traits. The addition of and predominance by the *L. plantarum* MDC^- culture under laboratory conditions exemplified one type of con-

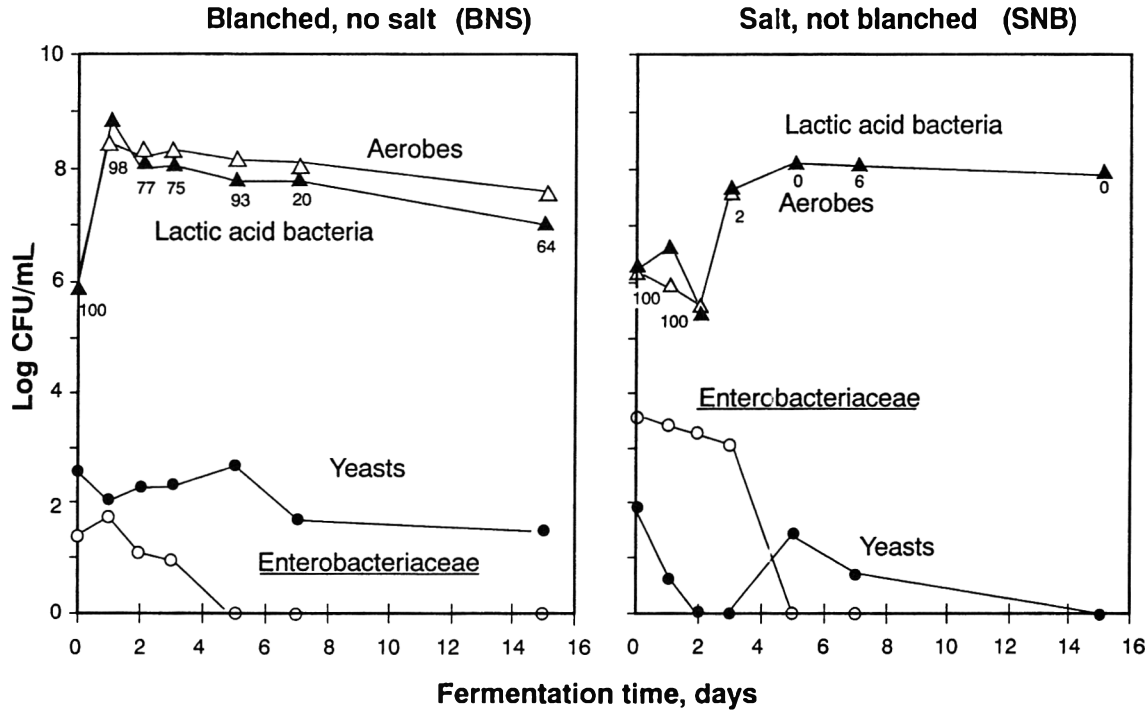

Fig. 2—Microbiological changes in cucumbers fermented in pilot tanks by two different procedures. The numbers that appear under data points for lactic acid bacteria refer to percentages of bacteria determined to be malate-negative. Microbial counts below log CFU/mL of 2 were estimates since the numbers of colonies per plate sometimes were <25.

Table 3—Quality of cucumbers fermented in pilot and laboratory fermentors[x]

		Brining treatment					
	Storage time	Firmness FPTY (kg)		Cure[z] (%)		Bloater index	
Fermentor	(months)	BNS	SNB	BNS	SNB	BNS	SNB
Pilot							
Top	1	7.1[b]	8.4[a]	60	70	29.0[b]	3.2
Middle	7	TS	8.3[a]	85	75	39.8[a]	10.0[cd]
	1	6.6[b]	8.1[a]	100	98	18.3[c]	1.5[d]
	7	4.0[c]	8.4[a]	100	95	7.8[d]	1.6[d]
Laboratory	1	7.6[b]	7.5[b]	50	55	0.0[d]	13.5[c]
	12	6.7[b]	8.2[a]	58	100	6.6[d]	4.4[d]

[x] Size 2B cucumbers, from the same lot, were fermented in 4,428L fiberglass, pilot tanks at a commercial firm, or in 19L plastic pails under laboratory conditions. Letters within firmness or bloater groupings designate statistically significant differences $P < 0.05$).

[y] BNS = blanched, no salt; SNB = salt, not blanched brining treatments; TS = too soft to determine firmness (<1.4 kg).

[z] Cure % was determined by subjective evaluation of entire lots and was not amenable to statistical analysis.

trolled fermentation. By elimination of purging requirements, economic disadvantages of the BNS system could be at least partially offset.

Clostridia have been shown to cause spoilage in acidified foods (Segmiller and Evancho, 1992). Even in fermented cucumbers, *Clostridium tertium* grew and contributed to the butyric spoilage of fermented cucumbers (Fleming et al., 1989). In that case, the cucumbers had been fermented to pH 3.7, and the acidity was 1% (as lactic acid). However, the salt concentration was only 2.3%, less than half the concentration used in commercial fermentations. Thus, further research is needed to determine the likelihood of spoilage of salt-free, fermented cucumbers by clostridia and other bacteria, and alternatives to salt for assuring microbial stability after fermentation and during extended storage. Addition of HCl to lower the brine pH to 3.3–3.5 after fermentation has been shown to provide the potential for microbial stability of brined cucumbers at 4% NaCl (Fleming et al., 1992b). Textural stability was reduced at lower pH values, but remained acceptable at pH 3.3–3.5

REFERENCES

Bell, T.A., Etchells, J.L., and Jones, I.D. 1955. A method for testing cucumber salt-stock brine for softening activity. USDA-ARS-72-5, 15 p.

Breidt, F. and Fleming, H.P. 1992. Competitive growth of genetically marked malolactic-deficient *Lactobacillus plantarum* in cucumber fermentations. Appl. Environ. Microbiol. 58: 3845–3849.

Buescher, R.W., Hudson, J.M., and Adams, J.R. 1979. Inhibition of polygalacturonase softening of cucumber pickles by calcium chloride. J. Food Sci. 44: 1786–1787.

Buescher, R.W., Hudson, J.M., and Adams, J.R. 1981. Utilization of calcium to reduce pectinolytic softening of cucumber pickles in low salt conditions. Lebensm. Wiss. Technol. 14: 65–69.

Daeschel, M.A., McFeeters, R.F., Fleming, H.P., Klaenhammer, T.R., and Sanozky, R.B. 1984. Mutation and selection of *Lactobacillus plantarum* strains that do not produce carbon dioxide from malate. Appl. Environ. Microbiol. 47: 419–420.

Daeschel, M.A., McFeeters, R.F., Fleming, H.P., Klaenhammer, T.R., and Sanozky, R.B. 1987. Lactic acid bacteria which do not decarboxylate malic acid and fermentation therewith. U.S. Patent No. 4,666,849.

Federal Register. 1987. Water quality criteria; availability of document. 52(195): 37654–37656.

Fleming, H.P., Daeschel, M.A., McFeeters, R.F., and Pierson, M.D. 1989. Butyric acid spoilage of fermented cucumbers. J. Food Sci. 54: 636–639.

Fleming, H.P., Humphries, E.G., and Macon, J.A. 1983. Progress on development of an anaerobic tank for brining of cucumbers. Pickle Pak Sci. VII: 3–15.

Fleming, H.P., McFeeters, R.F., and Daeschel, M.A. 1992a. Fermented and acidified vegetables. In *Compendium of Methods for the Microbiological Examination of Foods*, C. Vanderzant and D.F. Splittstoesser (Ed.), 3rd ed., p. 929–952, American Public Health Association, Washington, DC.

Fleming, H.P., McFeeters, R.F., Daeschel, M.A., Humphries, E.G., and Thompson, R.L. 1988. Fermentation of cucumbers in anaerobic tanks. J. Food Sci. 53: 127–133.

Fleming, H.P., McFeeters, R.F., and Thompson, R.L. 1987. Effects of sodium chloride concentration on firmness retention of cucumbers fermented and stored with calcium chloride. J. Food Sci. 52: 653–657.

Fleming, H.P., Thompson, R.L., Bell, T.A., and Hontz, L.H. 1978. Controlled fermentation of sliced cucumbers. J. Food Sci. 43: 888–891.

Fleming, H.P., Thompson, R.L., Bell, T.A., and Monroe, R.J. 1977. Effect of brine depth on physical properties of brine-stock cucumbers. J. Food Sci. 42: 1464–1470.

Fleming, H.P., Thompson, R.L., and Etchells, J.L. 1974. Determination of carbon dioxide in cucumber brines. J. Assoc. Offic. Anal. Chem. 57: 130–133.

Fleming, H.P., Thompson, R.L., Etchells, J.L., Kelling, R.E., and Bell, T.A. 1973. Bloater formation in brined cucumbers fermented by *Lactobacillus plantarum*. J. Food Sci. 38: 499–503.

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Fermentation of Lye-treated Carrots by *Lactobacillus plantarum*

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ABSTRACT

Sodium hydroxide treatment of peeled and trimmed carrots was investigated to rid them of natural epiphytic microflora that interferes with fermentation. It also formed a buffered brine by subsequent neutralization with acetic acid. Two lye concentrations (1% and 2% NaOH) and three treatment times (1, 15 and 30 min) were evaluated. Inoculation of *Lactobacillus plantarum* into NaOH-treated carrot brines resulted in a controlled fermentation yielding lactic acid almost exclusively. Percent carbon recovery varied from 83.7 to 148.3. More than 92% of available glucose and >85% fructose, but <13% sucrose, were utilised after 7 days fermentation. Texture and surface color (L*) of fermented NaOH-treated carrots were affected ($p < 0.05$) by NaOH concentration and treatment time. The product flavor was not adversely affected by alkaline treatment.

Key Words: carrots, fermentation, Lactobacillus, sodium hydroxide, lye treatment

INTRODUCTION

CARROTS have been subjected to lactic acid fermentation with good retention of color and texture, and the salt-sour flavor typical of fermented vegetables (Niketic-Aleksic et al., 1973). Starter cultures were useful to control the process and obtain more uniform products with good quality (Buckenhüskes, 1993). Fleming et al. (1983) used a prior pasteurization treatment to eliminate natural microflora and to thereby, favor establishment of the inoculum. Prior treatment did not have negative effects on sensory characteristics of fermented carrots (Rejano et al., 1994). However, it is rather costly to carry out, which would be a disadvantage on an industrial scale (Daeschel et al., 1987). Sodium hydroxide is widely used in processing vegetables for food. Its applications in chemical peeling of tomatoes and peppers (Anonymous, 1987), and for elimination of bitter flavor of green olives (Fernández-Díez et al., 1985) etc., are well known. Sodium hydroxide solutions have disinfectant properties (Harper, 1980) and this probably explains how pure culture fermentation was achieved in "Spanish style" green olives inoculated with starter *Lactobacillus plantarum* without prior thermal treatment (Montaño et al., 1993). Treatment of vegetables with NaOH prior to placing them in brine may, therefore, be a useful alternative to pasteurization, to achieve controlled fermentations. Subsequent neutralisation of the NaOH by acid added to the brine could result in formation *in situ* of a buffer. This could in turn produce a greater utilisation of fermentable material (Fleming et al., 1985). Given that the pectic substances are unstable in alkaline conditions (Codner, 1971) another possible advantage may be a decrease in texture. This would be an advantage if a product with softer texture than the raw material was required (e.g. for elderly consumers and/or those with dental problems, for dressings with a range of dishes, etc.).

Our objective was to determine the effects of different NaOH treatments on fermentation of carrots inoculated with a pure culture of *L. plantarum* and on sensory characteristics of final products.

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MATERIALS & METHODS

Carrots

Fresh carrots (cultivar "Nantesa") were obtained from a local market. The carrots were peeled, washed with tap water and cut into pieces of transverse section ≈4 × 8 mm and of length 15—25 mm, using a domestic electrical appliance.

Alkaline treatment and brining

The experiments were carried out in duplicate in ≈2 L jars containing 900g of carrots and 900g of liquid (NaOH solution or brine). Two concentrations of NaOH were studied: 0.96% and 1.92% (w/v). For each concentration three treatment times were tested: 5, 15 and 30 min. Immediately after treatment the alkaline solution was removed and brine was added (5% NaCl, w/v). The brine was acidified with glacial acetic acid in amounts equivalent—on a molar basis—to the quantity of NaOH absorbed by the carrots. Two replicate jars were left as controls. These contained brine without previous alkaline treatment nor acidification. Two hours after brine addition, while the pH of the samples was 5 to 7, all jars were inoculated with starter culture. *Lactobacillus plantarum* LP 91 from the Instituto de la Grasa culture collection, originally isolated from green olives fermenting brine, was used as starter culture. This was grown overnight at 32°C in MRS broth (Oxoid) containing 3% NaCl. Cells were harvested by centrifugation, washed and resuspended in saline. The initial population was 2×10^6 CFU/mL/jar, after inoculation of 1.7 mL of resuspended culture into each. A sterilized plastic bag was placed over the surface of each jar and distilled water was added to reduce risk of contamination and avoid contact with air. Fermentation was carried out in a room maintained at 25°C.

Buffer capacity

The buffer capacity β of a solution at any point on the pH scale is given by Van Slyke's formula (Pino and Valcárcel, 1975). Assuming buffer capacity of the system (carrots in brine) was only due to the buffer formed by added acetic acid and its conjugate base, the maximum buffer capacity β_{max} can be approximated by the formula:

$$\beta_{max} = 0.575\ C_t$$

where C_t is the normal concentration of acetic acid in each jar. This concentration was calculated assuming that carrots had a moisture content of 88% (Feinberg et al., 1991).

Sampling

Samples of brine from each jar were taken at 1, 2, 3, 4 and 7 days for microbiological analyses and determinations of pH and titratable acidity. Samples of carrots were taken at three times during processing: prior to alkaline treatment, after alkaline treatment and 7 days after brining. Samples (50g) taken before brining were mixed with an equal weight of 5% NaCl brine and homogenized using a mixer. Subsequently the solid part was separated by centrifugation at 16,000 × *g* and 4°C for 15 min using a Sorvall RC-5 superspeed refrigerated centrifuge (Du Pont Instruments, Newton, CT). The supernatant was frozen until analysis by GC and HPLC. Fermented samples were treated in a similar manner except they were mixed with the corresponding fermentation brines.

Microbiological analyses

Brine samples and their decimal dilutions were plated using a Spiral System model DS (Interscience, Saint Nom La Bretcche, France). *Enterobacteriaceae* were counted on Crystalviolet neutral-red bile dextrose

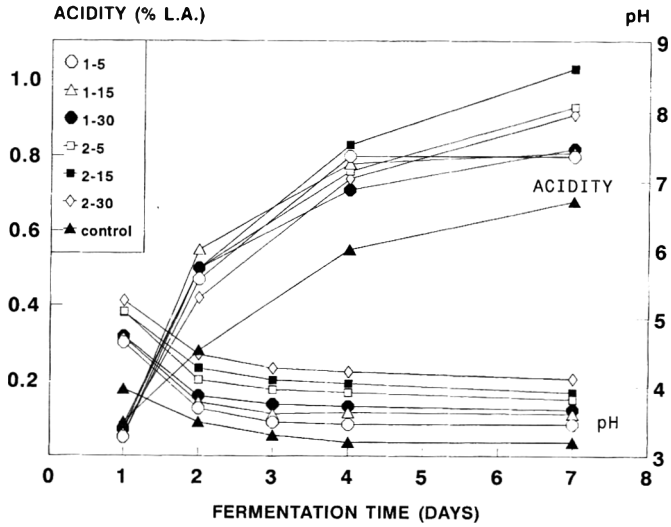

Fig. 1—Changes in pH and acidity (% as lactic acid) of brine during fermentation of carrots. (Means of duplicate fermentations. Coefficients of variation were: pH, 0.5% and acidity, 2.8%.)

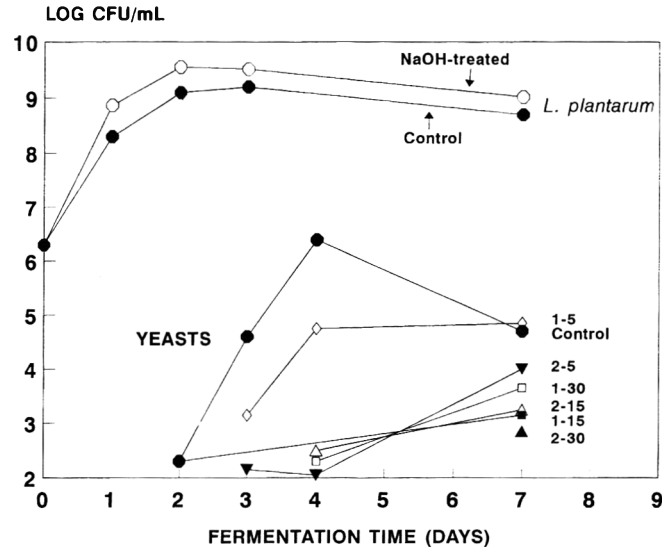

Fig. 2—Changes in *L. plantarum* LP91 and yeast counts in brine during fermentation of carrots. (Means of duplicate fermentations, except for *L. plantarum* LP91 growth curve of NaOH-treated samples where each point represents the mean of all samples. Coefficients of variation were: *L. plantarum*, 14.4% and yeasts, 90.8%.)

Table 1—Lye concentrations before and after alkaline treatment and maximum buffer capacity (β_{max}) of brine due to added acetic acid

Sample (≈ % lye-minutes)	NaOH (%) Initial	Final[a]	β_{max}[b]
1-5	0.96	0.68	0.021
1-15	0.96	0.52	0.033
1-30	0.96	0.52	0.033
2-5	1.92	1.36	0.043
2-15	1.92	1.20	0.055
2-30	1.92	1.04	0.067

[a] means of duplicate treatments.

[b] β_{max} = 0.575 C_t, where C_t is the normal concentration of acetic acid added in each jar assuming carrots have moisture content 88%. Native buffer systems for carrots not considered in calculation of maximum buffer capacity.

Table 2—Substrate concentrations before and after alkaline treatment

Compound (mM)[b]	Fresh carrots	NaOH-treated samples[a] 1-5	1-15	1-30	2-5	2-15	2-30
Sucrose	44.4	43.6	38.3	35.4	42.7	34.5	31.0
Glucose	27.2	27.2	20.0	20.0	22.8	21.1	17.8
Fructose	28.3	27.8	20.6	20.6	22.8	21.1	17.2
Malic acid	5.2	4.5	3.7	3.7	4.5	3.7	3.0

[a] Means of duplicate treatments, % lye-minutes.

[b] Concentrations in carrots/cover brine mixture (50/50, w/w).

agar (Merck); lactic acid bacteria on MRS agar (Oxoid) and yeasts on Oxytetracycline-gentamycin-glucose-yeast extract agar. Plates were incubated at 32°C for 48 or 72 hr.

Chemical analyses

The pH of brines was measured using a Crison 510 pH meter (Crison Instruments, S.A., Barcelona, Spain). Acidity was determined by titrating to pH 8.3 with 0.2N NaOH. Sodium hydroxide concentrations were determined by titration with 0.2N HCl with phenolphthalein indicator.

Sucrose, glucose, fructose and mannitol were analyzed by HPLC. The system consisted of a Hewlett-Packard Series 1050 liquid chromatograph equipped with a Rheodyne 7125 injector and a column heater, a Perkin-Elmer Model LC-25 refractive index detector and a Hewlett-Packard Model 3396 Series II integrator. An Aminex HPX-87C carbohydrate analysis column (Bio Rad Labs) held at 85°C was used. Deionized water was used as eluent at 0.7 mL/min. Samples (1 mL) were desalted by adding 2g of a mixed anion/cation resin (Serdolit MB-3, Serva Feinbiochemica Gmbh & Co., Heidelberg, Germany) which had previously been washed with deionized water and then dried under vacuum to remove excess water. An internal standard (1 mL 0.1% sorbitol) was also added for quantification by the internal standard method. Samples were shaken occasionally during a 60 min desalting period. A sample (≈1 mL) of the solution was centrifuged at 11,600 × *g* for 10 min and 20 µL were injected into the chromatograph.

Organic acids were also analyzed by HPLC using a Spherisorb ODS-2 (5 µm, 25 cm × 4 mm i.d., Teknokroma, Barcelona, Spain) column. The mobile phase was 0.2M KH_2PO_4, with pH adjusted to 2.4 using concentrated H_3PO_4 at 1.0 mL/min. Samples were diluted 1:5 with deionized water and then 5 µL concentrated H_3PO_4 were added to ensure that organic acids were in the protonated form. An aliquot (20 µL) was injected into the chromatograph after centrifugation at 11,600 × *g* for 15 min. Concentrations were calculated by comparison of peak heights with those of external standards for each compound.

Ethanol and methanol were analyzed using the headspace method described by Montañe et al. (1990).

Quality evaluation

Texture was measured using a Kramer shear compression cell coupled to an Instron Universal Testing Machine Model 1011. The cross head speed was 200 mm/min. Firmness of carrots in each jar was expressed as the mean of 10 replicate measurements. Each was performed on 2 pieces of carrot (total weight, 2.5g). Shear compression force was expressed as N/g of product.

Colorimetric measurements were carried out using a Color-View spectrophotometer (BYK-Gardner, Inc., Silver Spring, MD) with a measurement area of 11 mm diameter, 45° circumferential illumination and an observation angle of 0°. The apparatus was previously calibrated with a white standard (L* = 98.80, a* = −0.01, b* = −0.08). All measurements were done on the L*a*b* scale (CIE 1976) using illuminating conditions CIE type C, 10° observer. Results were expressed as the mean of five replicate measurements. Each was performed on one piece of carrot, avoiding the yellowish core.

Sensory evaluation of flavor preference was carried out by a 15-member taste panel. Each panelist assigned a number, 1, 2 or 3, to indicate 1st, 2nd or 3rd preferences. The numbers were compared with tables (Kramer and Twigg, 1970) to determine significance.

Statistical analyses

Statistical analyses of variance were carried out using the SPSS computer program (Norusis, 1985).

RESULTS & DISCUSSION

Effect of alkaline treatment on fermentation substrates

The concentration of NaOH in the lye decreased with time of treatment until it reached equilibrium (Table 1). For 0.96% and

Table 3—Fermentation effects 7 days after inoculation[a]

Sample (≈ % lye-min)	Substrates remaining (mM)[b]			Products formed (mM)[c]			Carbon rec. (%)[e]
	Sucrose	Glucose	Fructose	Lactic acid	Acetic acid[d]	Ethanol	
1-5	38.2 (87.6)	1.8 (6.5)	3.3 (11.9)	99.4	1.8 (43.3)	4.3	83.7
1-15	36.2 (94.5)	1.5 (7.4)	2.9 (14.2)	92.1	2.7 (60.0)	1.5	111.7
1-30	32.9 (92.6)	1.5 (7.5)	2.9 (14.5)	93.6	6.3 (63.3)	1.1	114.2
2-5	40.3 (94.8)	0.8 (3.4)	2.3 (10.1)	112.6	3.8 (78.3)	1.7	117.8
2-15	32.3 (93.7)	0 (0)	1.9 (9.3)	116.2	5.2 (101.7)	1.1	130.5
2-30	33.8 (100)	0 (0)	1.6 (9.1)	100.9	5.5 (115.0)	0	148.3
Control	39.4 (88.8)	6.9 (25.2)	8.3 (29.2)	88.4	6.0 (8.3)	51.7	136.5

[a] Values are means of duplicate samples and are obtained in the carrots/fermentation brine mixture (50/50, w/w).
[b] Substrate remaining = percentage of that present before fermentation in parentheses.
[c] In control, mannitol (1 mM; was also detected.
[d] Net increase in acetic acid over that present before inoculation. Total concentration in parentheses.
[e] Conversion of sugars and malic acid to fermentation products. Although CO_2 was not measured, we assumed that 1 mole CO_2 was produced for each mole of ethanol formed and that 1 mole CC_2 was produced for each mole of malic acid degraded.

Table 4—Texture and color of carrots[a] before and after fermentation

Sample (≈ % lye-min)	Texture (N/g)[b]		Color[b] after 7 days		
	Before fermentation[c]	After 7 days	L*	a*	b*
1-5	ND[d]	79.7	50.7	25.9	39.1
1-15	ND	64.2	50.9	29.3	40.3
1-30	51.2	58.6	48.9	27.5	38.7
2-5	ND	57.4	49.9	24.0	38.2
2-15	ND	53.5	49.4	26.5	37.8
2-30	28.8	41.7	46.0	23.2	33.9
Control	—	104.3	51.1	26.3	39.1

[a] Measurements in raw carrots were: texture = 114.5 N/g, L* = 55.0, a* = 29.8 and b* = 44.2.
[b] Means of duplicate samples.
[c] Analysis carried out immediately after alkaline treatment.
[d] ND = Not determined.

1.92% NaOH, equilibrium was reached between 5 and 15 min and between 15 and 30 min, respectively. The equilibrium value was in accordance with a simple dilution, assuming the moisture content of the carrots was 88%. As a result of treatment, a some of the fermentable material in the carrot was eliminated (Table 2). Particularly, in sample 2-30, 30—40% of the substrate initially present was eliminated. Acetic acid was detected in the carrots after each alkaline treatment at a concentration in the vegetable/brine mixture, of 5—7 mM. Montaño et al. (1993) reported a similar finding in "Spanish style" green olives. Another compound formed as a result of alkaline treatment was methanol. The concentration of methanol in the vegetable/brine mixture was very similar in the different treatment groups and was between 9 and 13 mM. Presumably the NaOH caused saponification of the ester groups of the pectins in the carrots, with liberation of methanol (Van Buren and Pitifer, 1992). Citric acid, which can be metabolised by *L. plantarum* (Hugenholtz, 1993), was found in very minor quantities in the raw material (0.5 mM in the vegetable/cover brine mixture). Its concentrations were practically the same after alkaline treatment.

Fermentation

The different amounts of acetic acid added to neutralize the amount of NaOH absorbed by the carrot tissue gave rise to notable buffering capacities (Table 1). Titratable acidity values were higher in the NaOH treated samples in comparison with the unbuffered control (Fig. 1). The higher the β_{max} value, the higher was the pH value during fermentation.

The growth of *L. piantarum* was similar in all NaOH-treated carrots, reaching up to 5.3×10^9 CFU/mL 48 hr after inoculation. The maximum population was slightly lower in the control, probably because the pH in this decreased at a faster rate (Fig. 2). *Enterobacteriaceae* were only detected in the control at 24 hr (2.7×10^5 CFU/mL). Carrots harbor an epiphytic microflora including *ca.* 10^5 CFU *Enterobacteriaceae*/g, which remains for 3—4 days during spontaneous fermentation in brine (Andersson et al., 1990; Rejano et al., 1994). Our results indicated that a proper inoculation reduced this population without eliminating it completely, whereas the lye treatment followed by inoculation eliminated it. The additional effect of acidification on this group of microorganisms should not be underestimated, however (McDonald et al., 1991). The process was partially effective in controlling the yeast population. This population was always higher in the control, although growth occurred in all samples. At day 4 yeast counts were 2.8×10^6 CFU/mL in the control, 2.9×10^5 in sample 1-5 and $\leq 4.3 \times 10^2$ in the other samples.

The concentrations of substrates and final products at 7 days fermentation were compared (Table 3). Consumption of sucrose was rather low. The presence of the buffer provoked a greater consumption of sugars in samples treated with NaOH than in controls, although in no sample were the sugars totally metabolised (except glucose in samples 2-15 and 2-30). The presence of residual sucrose after fermentation by *L. plantarum* can give rise to a secondary fermentation by yeasts in the packed product (Fleming et al., 1983). Thus, the use of some preservation method (pasteurization, additives) is necessary to achieve long-term stability. Malic and citric acids, although present in much smaller quantities than the sugars, were totally degraded in all samples. Lactic acid was the major product formed and acetic acid, although in very small amounts (<8 mM) was found in all samples. This contrasts with results of Fleming et al. (1983), who detected relatively high concentrations of acetic acid (≈100 mM) in carrots subjected to a pH-controlled fermentation with *L. plantarum*. In our study levels of ethanol in samples treated with NaOH (<5 mM) were notably lower than in the control (>50 mM). This illustrated the higher levels of yeast growth in the control throughout fermentation. Another product found in the control, although in small amounts (1 mM) was mannitol. The presence of this compound could be an index of growth of heterofermentative lactic acid bacteria (Fleming et al., 1985). Mannitol is a major product of fermentation (>54 mM) in carrots fermented spontaneously (Rejano et al., 1994). It was also detected by Andersson et al. (1990) in carrots inoculated with *L. plantarum* without treatment prior to inoculation.

Carbon recovery (Table 3) was 84% in sample 1-5, indicating that a portion of the substrates were transformed into compounds that were not measured. In the remaining NaOH-treated samples the carbon recovery values exceeded 100%. They were higher in those which received a stronger treatment (2-15 and 2-30). We hypothesized that part of the cell wall material, degraded as a result of alkaline treatment, was metabolised by *L. plantarum* with the production of lactic acid. In the control sample the other microorganisms that grow would contribute to the balance of fermentation. We could not explain the reasons for the high percentage recovery obtained.

Product quality

The determination of texture in samples 1-30 and 2-30, immediately after alkaline treatment, showed a marked decrease in texture in comparison with the raw material (Table 4). The greatest decrease occurred in sample 2-30. A degree of recovery of texture occurred in these samples after addition of brine. Ji-

menez (1993) reported a similar effect in "Spanish style" green olives. After 7 days fermentation the decrease in texture values varied between 30% and 64% compared with the raw material. In the control at the same time the decrease was only 8%. Analysis of variance on texture values showed a significant effect ($p < 0.05$) of NaOH concentration and treatment time. Note that the panelists, in general, considered the texture acceptable in all samples even in that with the poorest texture. Losses of firmness in NaOH-treated samples may be attributed to pectin depolymerization due to alkaline treatment. β-elimination splitting of the glycosidic bonds of pectin would be rapid at room temperature in alkaline conditions (Van Buren, 1979).

Alkaline treatment and fermentation caused a decrease in luminosity, L*, of the carrots and a decrease in the color parameters a* and b*, i.e. the product was less red and less yellow (Table 4). Analysis of variance on the colorimetric parameters at 7 days, showed a significant effect ($p < 0.05$) of NaOH concentration on L* and b*, while treatment time was only significant for L*.

In relation to flavor, the carrots treated with NaOH were generally well accepted by panelists. No differences ($p < 0.05$) occurred between samples treated with 1% NaOH or those treated with 2% NaOH. However, sample 1-15 was significantly preferred over the control. Higher amounts of acetic acid in samples treated with NaOH than in the control could have a positive effect on flavor. High volatile/nonvolatile acid ratios had a favorable effect on flavor of fermented cucumbers (Juhasz et al., 1974).

CONCLUSION

ALKALINE TREATMENT of peeled and cut carrots, followed by brine treatment with neutralization of excess NaOH (acetic acid) and inoculation with pure *L. plantarum* was adequate for controlling fermentation. This favored establishment of the inoculum as opposed to natural flora. At the same time this procedure modified the sensory characteristics (chiefly texture) of the carrots giving rise to interesting properties. This procedure could possibly be applied to other vegetables and may promote their consumption as fermented products. A detailed study would be necessary on each type product in order to determine effects of alkaline treatment.

REFERENCES

Anonymous. 1987. *A Complete Course in Canning and Related Processes*, 12th ed. Book III-*Processing Procedures for Canned Food Products*. The Canning Trade Inc., Baltimore, MD.

Andersson, R.E., Eriksson, C.E., Salomonsson, A.-C., and Theander, O. 1990. Lactic acid fermentation of fresh and stored carrot: chemical, microbial and sensory evaluation of products. Lebensm.-Wiss. u.-Technol. 23: 34–40.

Buckenhüskes, H.J. 1993. Selection criteria for lactic acid bacteria to be used as starter cultures for various food commodities. FEMS Microbiol. Rev. 12: 253–272.

Codner, R.C. 1971. Pectinolytic and cellulolytic enzymes in the microbial modification of plant tissues. J. Appl. Bacteriol. 34: 147–160.

Daeschel, M.A., Andersson, R.E., and Fleming, H.P. 1987. Microbial ecology of fermenting plant materials. FEMS Microbiol. Rev. 46: 357–367.

Feinberg, M., Favier, J.-C., and Ireland-Ripert, J. 1991. *Repertoire général des aliments*. Technique et Documentation-Lavoisier, Paris.

Fernández-Díez, M.J., Castro, R.de, Fernández, A.G., Cancho, F.G., Pellissó, F.G., Vega, M.N., Moreno, A.H., Mosquera, M.I.M., Rejano, L., Quintana, M.C.D., Roldán, F.S., García, P.G., and de Castro, A. 1985. *Biotecnología de la aceituna de mesa*. CSIC (Ed.), Madrid.

Fleming, H.P., McFeeters, R.F., Thompson, R.L., and Sanders, D.C. 1983. Storage stability of vegetables fermented with pH control. J. Food Sci. 48: 975–981.

Fleming, H.P., McFeeters, R.F., and Daeschel, M.A. 1985. The lactobacilli, pediococci and leuconostocs: vegetable products. In *Bacterial Starter Cultures for Foods*. S.E. Gilliland (Ed.), p. 97–118. CRC Press, Boca Raton, FL.

Harper, W.J. 1980. Sanitation in Dairy Food Plants. In *Food Sanitation*. R.K. Guthrie (Ed.), p. 154–192. AVI Publishing Co., Westport, CT.

Hugenholtz, J. 1993. Citrate metabolism in lactic acid bacteria. FEMS Microbiol. Rev. 12: 165–178.

Jiménez, A. 1993. Modificación de la pared celular de aceitunas (variedades Hojiblanca y Manzanilla) durante su elaboración. Doctoral Thesis. Universidad de Sevilla, Spain.

Juhasz, R.M., Molnár, E., and DeaK, T. 1974. Experiments on the pickling of vegetables by inoculation with pure cultures of lactic acid bacteria. Konzerv-es Paprikaipar. Special issue: 56. [Cited in Chavasit, V., Hudson, J.M., Torres, J.A., and Daeschel, M.A. (1991). J. Food Sci. 56: 462–465.]

Kramer, A. and Twigg, B.A. 1970. *Quality control for the Food Industry*, 3rd ed. Avi Publishing Co., Westport, CT.

McDonald, L.C., Fleming, H.P., and Daeschel, M.A. 1991. Acidification effects on microbial population during initiation of cucumber fermentation. J. Food Sci. 56: 1353–1356, 1359.

Montaño, A., Sánchez, A.H., and Rejano, L. 1990. Rapid quantitative analysis of headspace components of green olive brine. J. Chromatogr. 521: 153–157.

Montaño, A., Sánchez, A.H., and de Castro, A. 1993. Controlled fermentation of Spanish-type green olives. J. Food Sci. 58: 842–844, 852.

Niketic-Aleksic, G.K., Bourne, M.C., and Stamer, J.R. 1973. Preservation of carrots by lactic acid fermentation. J. Food Sci. 38: 84–86.

Norusis, M.J. 1985. *SPSSX Advanced Statistics Guide*. McGraw-Hill, New York.

Pino, F. and Valcárcel, M. 1975. *Equilibrios Iónicos en Disolución. Análisis Volumétrico*. Universidad de Sevilla, Spain.

Rejano, L., de Castro, A., Montaño, A., and Sánchez, A.H. 1994. Elaboración de zanahorias fermentadas. VI Simposium Andaluz del Alimento, Granada, Spain, March 22–23.

Van Buren, J.P. 1979. The chemistry of texture in fruits and vegetables. J. Texture Studies 10: 1–23.

Van Buren, J.P. and Pitifer, L.A. 1992. Retarding vegetable softning by cold alkaline pectin deesterification before cooking. J. Food Sci. 57: 1022–1023.

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Fleming, H.P., Thompson, R.L., and McFeeters, R.F. 1992b. Alternatives for assuring microbial and textural stability of fermented cucumbers. Food Technol. 46: 213.

McDonald, L.C., Shieh, D.-H., Fleming, H.P., McFeeters, R.F., and Thompson, R.L. 1993. Evaluation of malolactic-deficient strains of *Lactobacillus plantarum* for use in cucumber fermentations. Food Microbiol. 10: 489–499.

McFeeters, R.F. and Fleming, H.P. 1991. pH Effect on calcium inhibition of softening of cucumber mesocarp tissue. J. Food Sci. 56: 730–732, 735.

McFeeters, R.F., Thompson, R.L., and Fleming, H.P. 1984. Liquid chromatographic analysis of sugars, acids, and ethanol in lactic acid vegetable fermentations. J. Assoc. Offic. Anal. Chem. 67: 710–714.

McFeeters, R.F., Thompson, R.L., and Fleming, H.P. 1993. Malic acid analysis in cucumber juice and fermentation brines in the presence of interfering fructose. J. Food Sci. 58: 832–834.

Segmiller, J.L. and Evancho, G.M. 1992. Aciduric flat-sour sporeformers. In *Compendium of Methods for the Microbiological Examination of Foods*, C. Vanderzant and D.F. Splittstoesser (Ed.) 3rd ed., p. 291–297. American Public Health Association, Washington, DC.

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Mechanism of Surface White Discoloration of Peeled (Minimally Processed) Carrots During Storage

LUIS CISNEROS-ZEVALLOS, MIKAL E. SALTVEIT, and JOHN M. KROCHTA

ABSTRACT

A proposed mechanism of white discoloration development on peeled carrots included both physical and physiological responses to wounding. The physical response is a color change due to reversible surface dehydration. This study was performed at 2.5 and 10°C using a model system controlling relative humidity, at 33, 75 or 98%, and a commercial system with low-density polyethylene (LDPE) plastic film bags. The rate of surface discoloration increased with decreasing RH. When excess surface moisture was left on peeled carrots, rates of white development decreased sharply at all RH compared with a dewetted control. The same effects were observed on peeled carrots stored in LDPE bags. Carrots partially regained their original color when water-dipped, due to reversal of the physical response component.

Key Words: white discoloration, carrots, wounding response, phenolic metabolism

INTRODUCTION

PRECUT FRUIT AND VEGETABLE PRODUCTION is a growing industry. Peeled carrots (*Daucus carota*) represent an important component of the precut vegetable industry. They are produced from whole raw carrots washed, cut into ~5 cm long pieces, peeled, cooled to 1.5° C by hydrocooling with chlorinated water, dewetted (drained) and packaged in low-density polyethylene (LDPE) bags.

Minimal processing of fresh fruits and vegetables, such as trimming, peeling, cutting, slicing and other physical actions, causes injury and damage to tissues, affecting physiological activities and subsequently quality (Watada et al., 1990). Some problems related to cell disruption are leakage of nutrients, enzymatic reactions, mold growth, lactic acid fermentation, loss of texture, development of off-flavors and off-odors, and appearance defects (Carlin et al., 1990). All these factors limit the storage and market life of precut fruits and vegetables.

Surface white discoloration on peeled carrots during storage affects the produce quality and limits storage life. In published studies white appearance is considered a result of either surface dehydration of outer layers (Tatsumi et al., 1991, 1993; Avena et al., 1993a, b) or enzymatic activity and the formation of lignin (Bolin, 1991, 1992; Howard and Griffin, 1993, Howard et al., 1994) as a response to peeling.

When carrots are peeled, the periderm layer is removed, exposing inner tissues. Suberin is a characteristic component of periderm cell walls (O'Rear and Flore, 1983) and is associated with a wax complex (Soliday et al., 1979). Disrupted cell walls exposed to air by cutting or abrasion peeling consist mainly of cellulose, hemicellulose, lignin and other sugar polymers. Cellulose is hydrophilic in native form; lignin is considered hydrophobic, as reported in studies of wetting behavior in vessel walls in the xylem of plants (Laschimke, 1989). Suberized cell walls of the carrot periderm function as a primary barrier to mass transfer, and waxes of the suberin complex appear to cause the greatest impedance to water vapor diffusion (Soliday et al., 1979). Thus, removal of the periderm by peeling increases moisture loss from the carrot. Susceptibility to resulting white blush formation was reported to be influenced by temperature (Buick and Damoglou, 1987), relative humidity (Avena et al., 1993a), degree of peeling (Bolin and Huxsoll, 1991), and type of cutting surface (Tatsumi et al, 1991, 1993; Bolin and Huxsoll, 1991).

Our objective was to elucidate the mechanism by which white formation develops during storage of peeled carrots. Specifically, experiments were designed to determine what portion of peeled carrot white discoloration is attributable to the physical response of surface dehydration. Remaining white discoloration would be presumably due to physiological responses.

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MATERIALS & METHODS

Carrot samples

Peeled carrots (unknown cultivar) packaged in low-density polyethylene (LDPE) bags were obtained from a commercial processing plant in Bakersfield, CA., shipped over night under crushed ice to UCD and stored at 2.5°C. The study was performed on different lots from the same processing plant and ~24 hrs after the carrots were processed. Peeled carrots ~5 cm long and 12 to 16 g each were used.

Color evaluation

Color measurements of peeled carrots were made using a Minolta chromameter model CR200 (Minolta Camera Co, Japan), calibrated to a standard orange tile (L = 70.10, +a = 18.23, +b = 32.02). L, a and b values from the CIE (Commission Internationale de l'Eclairage) color scale (Gardner, 1975) were determined. Color measurement on each piece of peeled carrot was the average of 3 readings on different sites of the surface. Each piece of carrot was used as a replicate, using 10–20 replicates/treatment depending on the test. Results were expressed as whiteness index (W.I.), according to Judd (1963), and applied to peeled carrots (Bolin and Huxsoll, 1991).

A visual descriptive scale was used and related to the W.I. scale. The visual scale was defined as five levels of white color: nonwhite (0% white surface), slightly white (25% white surface), moderate white (50% white surface), severe white (75% white surface) and extreme white (100% white surface). To relate it with the W.I. scale, peeled carrots were grouped visually into these different levels of white development and measured with the Chroma-meter. A total of 10 to 20 replicates/group was used.

Wetting, dewetting and rewetting

Peeled carrots are usually dewetted by centrifuging (excess surface moisture removed) in commercial processing. For our study, all peeled carrots were wetted again by dipping in 200 ppm chlorinated distilled water to avoid microbial contamination. To obtain normal surface moisture (dewetted), the peeled carrots were dewetted by centrifuging with a salad spinner. To obtain initial excess surface moisture (wetted), peeled carrots were wetted, but not centrifuged, before storage. After treatment, peeled carrots were stored in controlled RH chambers or LDPE bags. Both dewetted and wetted peeled carrots initially had a moistened appearance. For some experiments, peeled carrots which had been stored were rewet by water-dipping in 200 ppm chlorinated distilled water and then draining.

Controlled RH chambers

Color changes of peeled carrots were studied at different relative humidities. Wetted peeled carrots were held in glass chambers at 10°C and conditioned at 33.5, 75.7 and 98.2% RH, obtained with saturated salt

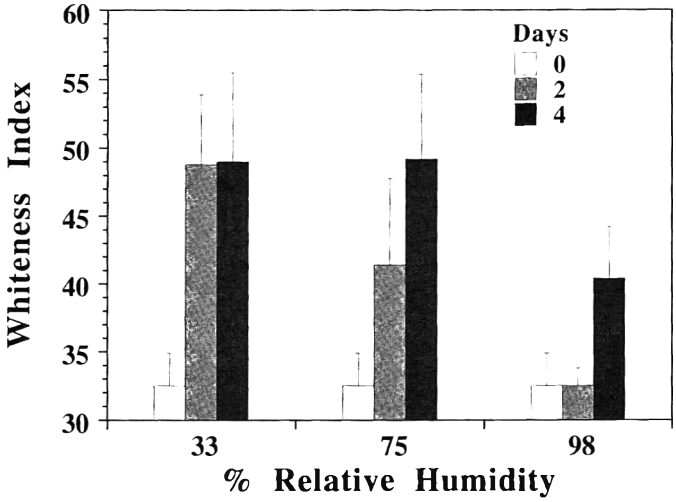

Fig. 1—Effect of relative humidity on Whiteness Index (W.I.) of wetted peeled carrots stored at 10°C. Bars indicate one-sided standard deviations.

Table 1—A visual description of white index values for peeled carrots

Description	White Index (W.I.)[a]
Non-white	32.6 ± 2.4
Slightly-white	38.4 ± 1.3
Moderate-white.	43.0 ± 1.8
Severe-white.	47.2 ± 1.7
Extreme-white.	50.9 ± 3.1

[a] Average values with standard deviations.

solutions of $MgCl_2$, NaCl and K_2SO_4 (Fisher Scientific Co., Fair Lawn, NJ), respectively. Preparation was according to ASTM method (1991). The saturated salt solutions were prepared as follows: $MgCl_2$ (500g salt/62.5mL water), NaCl (500g salt/150mL water) and K_2SO_4 (500g salt/273 mL water). A Solomat hygrometer (Solomat Corp., Stamford, CT) was used to measure relative humidity. The experiments were performed mostly without air movement to simulate conditions inside plastic packages. A fan inside each chamber was used only for short periods right after opening and closing the chamber lid to quickly re-equilibrate the RH in the chamber after color measurements had been made. Peeled carrots were placed over a stainless steel metal screen above the saturated salt solution (2.5 cm), avoiding contact between pieces. Ten peeled carrots were used as replicates for each treatment.

Effect of rewetting. Dewetted peeled carrots were stored for 4 or 8 days at relative humidities of 75.7 and 98.2% RH to induce white development. L, a and b values of the peeled carrots were measured and the W.I. values calculated. Carrots were then water-dipped to rewet. The rewetting procedure was performed by dipping 10 peeled carrots (~122 g total) in a glass vessel containing 500 mL of chlorinated distilled water (200 ppm) stirred at room temperature (~23°C). After draining carrots were measured for color. After each measurement, carrots were again dipped in fresh chlorinated distilled water. Results were reported as W.I. related to total dipping time.

Effect of excess surface moisture. Wetted and dewetted peeled carrots were stored at 75.7 and 98.2% RH for a total of 8 days. Color measurements were performed at days 0, 2 and 4. At day 4, ten carrots were rewetted for 10 min and the color measured. The carrots were treated again by wetting or dewetting in a manner identical to day 0. Carrots were then placed back in the chambers and color measurements were performed at days 6 and 8. Finally at day 8, carrots were water dipped for another 10 min and the color measured.

Studies in LDPE bags

White discoloration on peeled carrots was also studied using a commercial packaging system at 2.5 and 10° C, placing 250g of carrots (~20 carrots) in each LDPE plastic bag. Plastic films of 1.5 mil thickness and area of 435 cm^2 (14.5 cm × 15 cm × 2 sides) were used for making the bags. These films had an average water vapor permeability of 9.41 × 10^{-6}g kpa^{-1} hr^{-1} m^{-1} at 20°C, measured with the cup method according to ASTM method (1989). The relative humidity of the storage room was 75 ± 5% with air velocity of 20 m min^{-1}. Plastic bags were sealed using a manual heat sealer (PGC Scientific, Gaithersburg, MD).

Both wetted and dewetted peeled carrots were placed in plastic bags at 10°C. Evaluations were done periodically, monitoring 20 peeled carrots each time/treatment. Two bags/treatment were used. Bags were opened each time color measurements were taken; afterwards, the carrots were placed in new bags and sealed again. Results were reported as W.I. change related to total storage time.

Dewetted peeled carrots were stored at 2.5°C in LDPE bags for 2 and 4 wk. After these periods, 10 peeled carrots were measured for levels of white appearance and then water dipped in chlorinated distilled water (200 ppm) stirred at room temperature. After draining, carrots were measured for color. After each measurement, carrots were again dipped in fresh chlorinated distilled water. Results were reported as W.I. as related to total dipping time.

Statistical analysis

Statview 4.0 was used for statistical analyses (Abacus Concepts, Berkeley, CA). Analysis of variance and Fisher PLSD multiple-comparison tests were performed.

RESULTS & DISCUSSION

White index-sensory scale relationship

The whiteness index scale (W.I.) was related to a visual descriptive scale to achieve a better understanding of W.I. data (Table 1). From this relation, we could define some general limits: a non-visible white as a W.I. of 32.6 ± 2.4, and a moderate white as a value of 43.0 ± 1.8 W.I. Higher values indicated that the peeled carrots had reached their storage life limit. Visual ratings were reported by Bolin and Huxsoll (1991), Bolin (1992) and Avena et al. (1993a, 1993b), but were not directly related to the W.I. scale.

Relative humidity association with color change

Avena et al. (1993a) reported a W.I. development dependence on relative humidity for peeled carrots. We confirmed their results, observing different rates of W.I. appearance on peeled carrots through time at three relative humidities (Fig. 1). These results were related to water loss from the surface, considering the inverse relation with % RH. Rooke and Van den Berg (1985) showed that when whole carrots were exposed to 100% RH, they slowly absorbed some moisture; while at 96–99% RH, whole carrots lost moisture, depending on RH of the air. The equilibrium relative humidity, which is the RH of air in equilibrium with the tissue (no net moisture transfer between tissue and air), is about 99.6–99.8% for whole carrots (Rooke and Van den Berg, 1985). The driving force for moisture loss would be the vapor pressure difference (VPD) between the surrounding air and the peeled carrot surface. The VPD is dependent on temperature and % RH. Buick and Damoglou (1987) observed a temperature effect on color change, which could be related to this dependence of VPD on temperature.

Reversible color change

Dewetted peeled carrots exposed to 75 and 98% RH for 4 days had a W.I. corresponding to a severe white (Fig. 2). When the carrots were dipped into water (rewet), an exponential decay relation between W.I. and water dipping time was observed. After ~4 min of water exposure, a value of W.I. was reached which corresponded to non-visible white color. W.I. continued to decrease with increased dipping time, with no significant ($p < 0.05$) additional reduction after 8 min. Peeled carrots held for 8 days under similar storage conditions also presented lower W.I. value after water dipping, with no ($p < 0.05$) additional reduction after 20 min. The minimum values of W.I. reached in both cases were higher than those corresponding to fresh carrots at day 0. This partial reduction of W.I. values was 75 to 90% of the original color change.

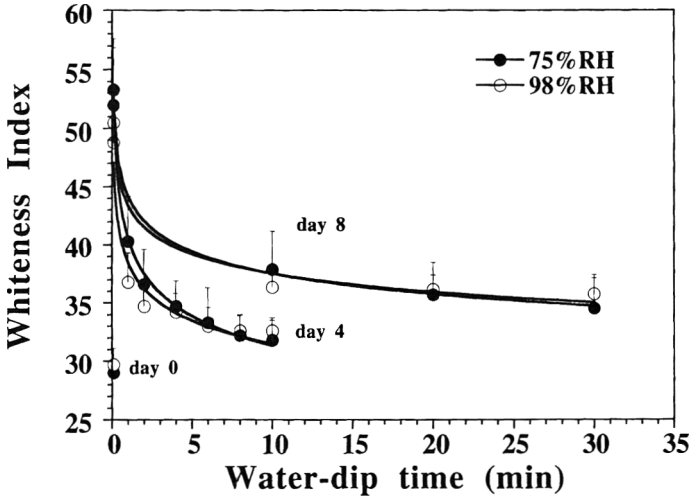

Fig. 2—Effect of water dip time on W.I. of dewetted peeled carrots previously stored for 4 and 8 days at different relative humidities and 10°C. Bars indicate one-sided standard deviations.

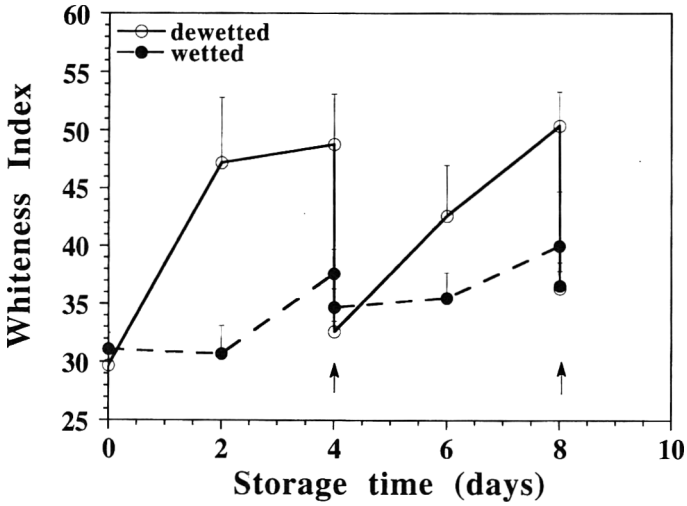

Fig. 3—Effect of initial surface moisture and storage time on W.I. for peeled carrots stored at 98% RH and 10°C. Arrows indicate a ten minute water dip. Bars indicate one-sided standard deviations.

The partial reversibility was likely due to rehydration of the dried surface and the filling with water of spaces between disrupted cell walls or debris. A moistened surface reduces reflectance of light, making more apparent the deep orange color beneath the surface. The cell wall debris of the surface would appear translucent in the presence of water. When the surface of peeled carrots dries, it scatters reflected light, causing the white appearance.

This reversible color change suggests a surface dehydration mechanism and is most likely what Avena et al. (1993a) reported as depending on %RH. The degree of peeling and the type of cutting surface associated with color change (Bolin and Huxsoll, 1991; Tatsumi et al., 1991) could be explained in part by this observation. More disrupted cell walls on a dried surface cause more irregular surface areas. This increased area once dried would increase light scattering and thus white appearance.

Initial surface moisture

Avena et al. (1993a) showed that during surface dehydration, a lag time was observed before W.I. increased. This lag time would depend on the initial amount of water present on the surface of the peeled carrots. After placing wetted and dewetted

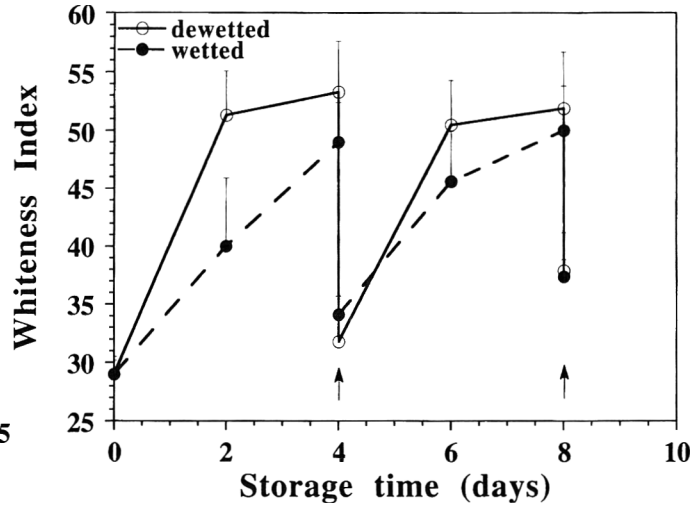

Fig. 4—Effect of initial surface moisture and storage time on W.I. for peeled carrots stored at 75% RH and 10°C. Arrows indicate a ten minute water dip. Bars indicate one-sided standard deviations.

peeled carrots at 98% RH, different rates of white index development could be observed (Fig. 3). For previously-dewetted peeled carrots, the white development was always higher. After 4 days, the difference in white development was still observed, although in that case initially-wetted peeled carrots had reached a slightly-white stage.

Water dipping (rewetting) caused peeled carrots to regain an orange color, thus a low W.I. value. When placed back in the chambers, the white development was again induced under similar rates, reaching after another 4 days values similar to those reported before. At 75% RH, this effect was observed again (Fig. 4). In that case, the difference between wetted and dewetted peeled carrots was not as notable as at 98% RH. This could be due to the higher driving force or water vapor pressure difference at 75% RH, inducing a higher rate of surface water loss.

The presence of excess surface moisture could help extend the lag time observed before white discoloration appears. Treatments claiming to control enzymatic activity, such as aqueous acid or basic dip treatments (Bolin and Huxsoll, 1991; Bolin, 1992) or steam treatments (Howard et al., 1994) were most likely maintaining a moisture layer over disrupted cell walls. This would delay observed white development, but basically due to an increase of lag time as reported by Avena et al. (1993).

Irreversible color change

Dewetted peeled carrots exposed to 75 and 98% RH and then water dipped did not regain totally their original color (Fig. 2). The same was observed for wetted carrots (Fig. 3, 4). These increased W.I. values were due to an irreversible color change, and according to our observations these values increase with time. It has been reported that when wounding occurs, phenolic metabolism was activated in carrots, inducing lignification of outer cells (Bolin and Huxsoll, 1991; Howard and Griffin, 1993). This physiological response has been proposed as the cause of white discoloration. We propose that the irreversible color change we observed could be related to the irreversible physiological response to wounding reported in other studies. The mechanism that could explain the irreversible color change may be related to the wetting characteristics of lignin, the light reflectance of lignin, or both. Considering that lignin is hydrophobic (Laschimke, 1989), the irreversible W.I. component possibly shows a non-complete rehydration of the outer layers (initially more hydrophilic) during water dipping. Also the lignification process may increase light reflectance and thus white development. From our observations, the irreversible color

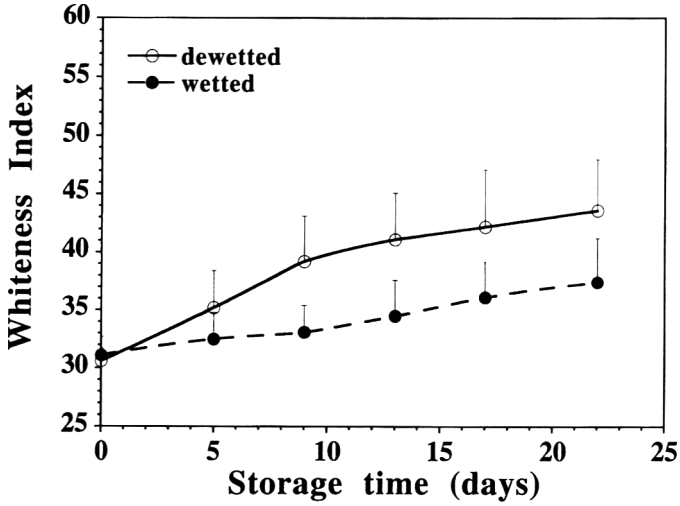

Fig. 5—Effect of storage time on W.I. of wetted and dewetted peeled carrots stored in LDPE bags at 10°C. Bars indicate one-sided standard deviations.

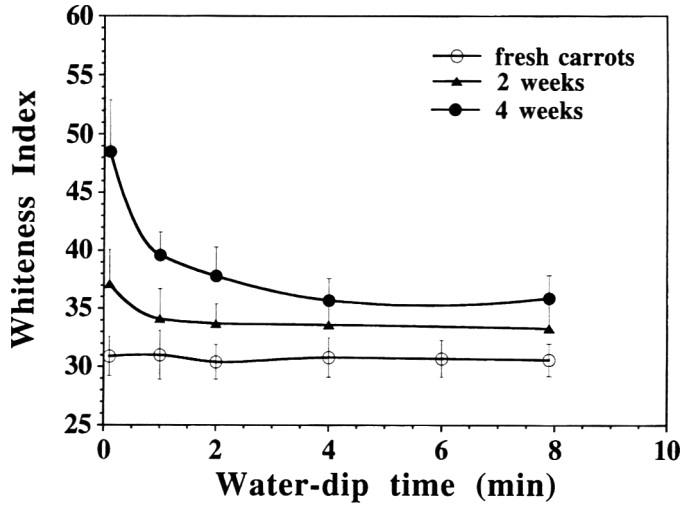

Fig. 6—Reversible and irreversible color change of dewetted peeled carrots previously stored for 2 and 4 wk at 2.5°C in LDPE bags compared to fresh carrots. Bars indicate one-sided standard deviations.

change appears to be independent of storage RH or initial amount of moisture on the peeled carrots surface (Fig. 3, 4). Howard and Griffin (1993) quantified the increase of lignin and phenolic compounds and the increase in phenylalanine ammonia-lyase (PAL) activity on carrot sticks, and correlated it with an increase in total W.I. They observed that upon reducing ethylene, the whiteness index did not decrease. The irreversible color change may be associated with factors related to the phenolic metabolism pathway. Oxygen, ethylene, carbon dioxide, wounding severity (Bolin and Huxsoll, 1991; Tatsumi et al., 1991), cultivar, temperature (Buick and Damagon, 1987), and other factors that could affect gene reading or enzyme activity might influence this response.

Thus, our results suggest that the W.I. development on peeled carrots has two components. First, a physical response component due to surface moisture loss, manifested by the partial reversibility of W.I. once water-dipped. Second, a possible physiological response component due to lignification (Bolin and Huxsoll, 1991; Howard and Griffin, 1993) and appearing as an irreversible increase of W.I. after water dipping. In commercial packaging systems, where fluctuations in storage temperature may occur and water condensation inside bags takes place, localized surface rehydration can occur. The result would be variations in white development among carrots packaged in a bag and between bags in the same storage facility. Considering that potentially the reversible white color change has more effect than the irreversible component, the physiological response may be masked. Studies on enzymatic effects on color change (Bolin and Huxsoll, 1991; Bolin, 1992; Howard and Griffin, 1993; Howard et al., 1994) most likely also have been observing dehydration effects on color.

Carrots stored in LDPE bags

The RH in packaged vegetables and fruits can reach high values. Shirazi and Cameron (1992) reported a relative humidity of 98% in LDPE plastic-film-packaged tomatoes (2 mil thickness at 20°C). The RH inside the plastic bags would also be dependent on the storage room RH and the plastic film permeance (Saguy and Mannheim, 1975). Any other factor, such as cultivar or growing conditions, that affects fruit skin resistance to water loss, may contribute to variations in % RH (Shirazi and Cameron, 1992).

Peeled carrots have very low water vapor resistance (Avena et al., 1993b), indicating high moisture loss. Considering that there is a high relative humidity inside the bags, a small vapor pressure difference between the inside package air (that surrounds the carrots) and the carrot surface would be enough to cause loss of surface water. Using a packaged system where the plastic film defines the rate of water loss from the peeled carrots, similar behavior to that observed in chambers controlled at 98% RH was obtained (Fig. 5). Wetted peeled carrots had a lower rate of W.I. development compared to a control that had been dewetted before packaging. This was evident after 14 days, and the difference was maintained as time increased; but ultimately the W.I. reached similar values (data not shown). In that case, the lag time was extended and the white appearance delayed.

Dewetted peeled carrots stored in LDPE bags at 2.5°C showed reversible and irreversible W.I. change components once water dipped (Fig. 6). In the 2 week storage period, the reversible color change represented 50% of the total change in color; while for the 4 wk storage the reversible color change was ~72% of the total. In both cases, white discoloration was observed due to the physical response.

CONCLUSIONS

A PROPOSED MECHANISM of white discoloration development on peeled carrots includes both physical and physiological responses to wounding. The physical response is reflected in a color change due to surface dehydration which is reversible. As time passes, a possible physiological response occurs involving activation of phenolic metabolism and production of lignin reflected by an irreversible color change. In general, white development due to the physical response increases with lower RH and with time. Initial treatments using excess surface moisture would reduce the rate of white development. In commercial systems, different factors affecting mass transfer should be considered and evaluated. These include relative humidity and air velocity in storage rooms, temperature fluctuations, ratio of weight of produce to film area, thickness and permeability.

REFERENCES

ASTM, 1989. Standard test methods for water vapor transmission of materials. *Annual Book of ASTM Standards*. Designation: E96-80.

ASTM, 1991. Standard practice for maintaining constant relative humidity by means of aqueous solutions. *Annual Book of ASTM Standards*. Designation: E104-85

Avena-Bustillos, R., Cisneros-Zevallos, L., Krochta, J.M., and Salveit. M.E. 1993a. Application of casein-lipid edible film emulsions to reduce white blush on minimally processed carrots. Postharvest Biology and Tech. 4: 319–329.

Avena-Bustillos, R.; Cisneros-Zevallos, L.; Krochta, J.M., and Salveit. M.E. 1993b. Optimization of edible coatings on baby carrots to reduce blush response surface methodology. Transactions of the ASAE. 36: 801–805.

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Sulfite and Starch Affect Color and Carotenoids of Dehydrated Carrots (*Daucus carota*) during Storage

Y.P. ZHAO and K.C. CHANG

ABSTRACT

Sulfite and starch treatments influenced the quality of dehydrated carrots. Initially, redness of dehydrated carrots was high in sulfite-treated samples. Redness and tota. α- and β-carotenes decreased as storage time increased in all treatments. Starch-treated samples had a slower rate of carotene loss, and retained more ($P < 0.05$) red color than did control and sulfite-treated samples. When residual sulfite in dehydrated carrots decreased to ≈≤200 ppm, its effectiveness in protecting carotene was not notable.

Key Words: carrot dehydration, sulfite, starch, carotenoids

INTRODUCTION

DEHYDRATED CARROTS are important components in various products in the U.S. food industry. Deterioration of chemical and physical properties of dehydrated carrots causes major changes in their quality and stability during storage. Baloch et al. (1981) reported that sulfite treatment, using 0.6% sodium metabisulfite for 6 min, retarded carotenoid breakdown, inhibited lipid oxidation, and decreased discoloration of dehydrated carrots. The reactions of sulfite in foods are complex. However, reaction of sulfite with carbonyl groups, reducing sugars, and disulfide bonds in proteins and enzymes may be responsible for the effects in dehydrated carrots (Taylor et al., 1986).

The maximum allowable sulfite residue concentration for dehydrated carrot products in the U.S. is 200 ppm (Federal Register, 1988). High concentrations of sulfite residues in foods may affect certain consumer's health (Bhagat and Lockett, 1964; Inoue et al., 1972; Taylor et al., 1986). People with asthma are often allergic to sulfite in foods. For some individuals, severe bronchospasms and faintness may result from sulfite ingestion (Taylor et al., 1986). Little information is available on effects of low level sulfite treatment (residue concentration <200 ppm) on quality of dehydrated carrots.

Coating carrots, which had been steam blanched for 5 min, in a hot starch solution (2.5%, at 77°C for 1 min) prevented carotenoid loss and extended storage life of dehydrated carrots (Baloch et al., 1986). Starch coating gave a surface coverage and, hence, protected carotene effectively by isolating oxygen from carrots (Baloch et al., 1986). Starch treatment of carrots while boiling to achieve blanching and coating effects simultaneously has not been reported.

Our objective was to compare effects of sulfite treatments at various concentrations, and starch treatment at the boiling temperature on quality of dehydrated carrots.

MATERIALS & METHODS

Sulfite treatment

Carrots (Bunny-Luv brand from California) were purchased from a local grocery store in Fargo, ND. They were peeled and diced (1 cm cubes). Carrots (≈150 g) were blanched with steam at 100°C for 3 min. The steam-blanched carrots were treated with two levels of sodium bisulfite ($NaHSO_3$): 0.05% (w/v) and 0.2% (w/v). Carrots were soaked in the sodium bisulfite solution (450 mL) at 65°C for 8 min and dehydrated. As a control carrots were soaked in distilled water at 65°C for 8 min and dehydrated. A single layer of blanched carrots was placed on a screen (U.S. standard No. 8) and dehydrated in an air oven at 90°C for 2 hr and at 70°C for 4 hr. Sulfite and control treatments were carried out in four replicates.

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Starch treatment

Diced carrots (150g in four replicates) were boiled for 3 min in 2.5% corn starch solution (Geo. T. Walker and Company, Inc., Minneapolis, MN). The dehydration method was the same as that of sulfite-treated carrots.

Storage and quality evaluation

The quality of sulfite- and starch-treated dehydrated carrot samples was assessed at 4-mo intervals for a total of 12 mo. Dehydrated carrots from each treatment were pooled, mixed, placed into plastic containers, sealed in tin cans (size 303 × 406) to prevent moisture penetration, and stored at ambient temperature (22–26°C). The color of dehydrated and rehydrated carrots, rehydration ratio, α-, β- and total carotenes, and residual sulfite were determined immediately after drying and after storage for 4, 8, and 12 mo.

Moisture contents of dehydrated carrots before and after storage were determined (AOAC, 1990). Residual sulfite concentrations of both dehydrated carrots and rehydrated carrots, were determined as sulfur dioxide, using distillation followed by redox titration of sulfite ion by iodine, (DeVries et al., 1986). Carotene content was determined according to the high performance liquid chromatographic method of Bushway (1985). Rehydration ratio was determined after cooking 4g of dehydrated carrots in 250 mL distilled water for 30 min. Rehydration ratio was calculated by dividing the drained rehydrated weight by the dry weight of the carrots. The surface color of dehydrated and rehydrated carrots was measured, using a Gardner Tristimulus colorimeter equipped with a reflection optical system (model XL-23, Gardner Lab. Inc., Bethesda, MD). The colorimeter was calibrated with a standard white tile ($L = 91.94$, $a_l = -1.03$, $b_l = 1.14$).

Statistical analyses

The 4 × 4 factorial experimental design was used to examine the four treatments (control, 0.05% sulfite, 0.2% sulfite, and 2.5% corn starch) and four periods of storage (0, 4, 8, and 12 mo). Experimental variables were rehydration ratio, sulfite residue content, color (L, a_l, and b_l), and carotene contents (α, β, and total carotene). All variables were subjected to analysis of variance, using the general linear model procedure (SAS Institute, Inc., 1990). Mean differences among treatments and storage periods were tested for significance using Duncan's multiple range test (SAS Institute, Inc., 1990).

RESULTS & DISCUSSION

THE 2.5% CORN STARCH-TREATED CARROTS had a lower ($P < 0.05$) rehydration ratio than controls, 0.05% sulfite-treated, or 0.2% sulfite-treated carrots (Table 1). The rehydration ratio of 2.5% corn starch-treated carrots was 20% lower than that of the control, 0.05% sulfite-treated, and 0.2% sulfite-treated carrots through 12 mo. Polysaccharide gel absorbed on the cell walls probably caused the low rehydration ratio of the starch-treated sample. When carrots are boiled with starch solution, the starch may penetrate into the carrot tissue and change the texture and rehydration (Mudahar, 1989).

Table 1—Effects of sulfite and starch treatments on rehydration ratio of dehydrated carrots during storage[a]

Month	Control	0.05% S	0.2% S	2.5% CS
0	6.9a,(fg)	7.0a,(e)	6.9a,(e)	5.6b,(fg)
4	7.1a,(ef)	6.1c,(g)	6.6b,(f)	5.7c,(f)
8	7.4a,(e)	6.7b,(f)	6.7b,(ef)	6.0c,(e)
12	6.7a,(g)	6.8a,(ef)	6.9a,(e)	5.4b,(g)

[a] Means represent two determinations. S = sulfite and CS = corn starch. Means in the same row with different letters a, b, c, or d and same column with different letters (e), (f), (g), or (h) differ ($P < 0.05$).

Dehydrated carrots with a higher rehydration ratio have a higher product yield. Dehydrated carrots with high rehydration ratio have softer texture after rehydration than dehydrated carrots with low rehydration ratio. A high rehydration ratio is not always advantageous for every food application. Dehydrated carrots with a high rehydration ratio are preferred for cake or muffin mixes because a high moisture holding increases final product tenderness. However, dehydrated carrots with low rehydration ratio are preferred for stew mixes because their texture can hold up better after cooking.

Eusebio et al. (1980) reported that carrots pretreated with sulfite, starch, or blanched in boiling water resulted in rehydration ratios of 5.2–5.7, 8.2–8.5, and 4.2–4.3, respectively. The starch treatment had a higher rehydration ratio than did the sulfite and control groups. The differences between the results of our study and those of Eusebio et al. (1980) may be due to differences in processing methods. In the starch research of Eusebio et al. (1980), carrots were steam blanched and dipped in 2.5% corn starch solution for 1 min. In our study, however, carrots were directly cooked in 2.5% boiling corn starch solution for 3 min. In the sulfite treatment, Eusebio et al. (1980) blanched carrots in boiling 0.2% sodium metabisulfite for 2 to 3 min. In our study, carrots were steam-blanched and dipped for 8 min in 0.05% or 0.2% sodium bisulfite solution.

Baloch et al. (1981) observed that the rehydration of freshly dehydrated carrots restored only 46.2–60.2% of initial weight. However, rehydration of dehydrated carrots that had been stored for 180 days at 37°C in sealed tin cans restored only 2.2–13.7% of initial weight. The loss of rehydration was due to changes in macromolecular components, including cellulose, pectin, hemicellulose, and protein, which were adversely affected during pretreatment, dehydration and storage (Weier and Stocking, 1949).

The 0.2% sulfite-treated carrots had higher ($P < 0.05$) residual sulfite than 0.05% sulfite-treated carrots in both dehydrated and rehydrated carrots (Table 2). The residual sulfite content of 0.2% sulfite-treated dehydrated carrots decreased from 1,460 to 727 ppm during the 12-mo period. Rehydration greatly reduced residual sulfite content in carrots. The loss during rehydration might be due to the combination of cooking and sulfite leaching into the cooking water. Compared to the beginning (0-month) dehydrated sample, the total residual sulfite content of 0.2% sulfite-treated dehydrated carrots decreased by 10, 21, and 50% after 4-, 8-, and 12-mo storage, respectively. Compared to the rehydrated 0-month sample, sulfite content of the 0.2% sulfite treated sample decreased by 2, 26, and 87%.

Residual sulfite of 0.05% sulfite-treated carrots decreased from 223 to 18 ppm in dehydrated carrots and from 40 to 5 ppm in rehydrated carrots during 12-mo storage (Table 2). The total sulfite residue decreased by 68, 80, and 92% in dehydrated carrots and decreased further by 27, 58, and 86% in rehydrated carrots during the 4-, 8-, and 12-month periods, respectively. These levels of sulfite in rehydrated carrots were below the limits (<200 ppm) for dehydrated vegetables (Federal Register, 1988). Sulfite content decreases during storage as a result of complex reactions with food components. Several reactions, including those with carbonyls, reducing sugars, and proteins, have been described (Taylor et al., 1986).

Orange color is related to carotenoid pigments (Borchgrevink and Charley, 1966). The effects of sulfite and starch treatments were compared on color of dehydrated carrots (Table 3). The L value of 2.5% corn starch-treated carrots was lower ($P < 0.05$) than that of the control, 0.05% sulfite-treated, and 0.2% sulfite-treated carrots. This pattern was maintained after 4- and 8-month periods.

Table 2—Residual sulfite contents of sulfite-treated dehydrated carrots[a]

Month	0.05% sulfite treatment	0.2% sulfite treatment
	Dehydrated carrot, ppm	
0	222.7a	1460.2a
4	70.7b	1310.6b
8	45.5c	1160.4c
12	18.1d	726.8c
	Rehydrated carrot, ppm	
0	40.5a	147.2a
4	29.7b	144.9a
8	16.9c	108.5b
12	5.1d	18.7c

[a] Means represent two determinations. Data are on a dry weight basis. Probability that treatments differ. Means in the same column for each type of product with different letters a, b, c, or d differ ($P < 0.05$).

Table 3—Effects of sulfite and starch on color of dehydrated carrots during storage[a]

Month	Control	0.05% S	0.2% S	2.5% CS
	L (lightness)			
0	36.7a,(f)	37.3a,(e)	37.5a,(e)	32.4b,(f)
4	36.9a (f)	37.4a,(e)	37.9a,(e)	33.6b,(e)
8	37.4a (f)	37.8a,(e)	38.0a,(e)	32.1b,(fg)
12	40.3a (e)	37.4b,(e)	38.0b,(e)	33.3c,(ef)
	a_L (redness)			
0	32.2a (e)	34.3a,(e)	34.3a,(e)	32.8a,(e)
4	31.6b,(e)	32.4a,(f)	32.4a,(f)	31.5b,(e)
8	27.1b,(f)	29.4a,(g)	30.9a,(g)	29.5a,(f)
12	24.8c (g)	26.9b,(h)	30.5a,(g)	29.8a,(f)
	b_L (yellowness)			
0	19.4a,(e)	19.4a,(e)	19.6a,(e)	17.2b,(e)
4	18.8a,(ef)	19.4a,(e)	18.8a,(e)	16.9b,(e)
8	18.1b,(f)	18.4b,(f)	19.2a,(e)	16.0c,(f)
12	19.3a,(e)	18.5a,(f)	19.2a,(e)	16.4b,(ef)

[a] Means represent two determinations. S = sulfite and CS = corn starch. Means in the same row with different letters a, b, c, or d and same column for each attribute with different letters (e), (f), (g), or (h) differ ($P < 0.05$).

The initial a_L values of 0.05% sulfite-treated and 0.2% sulfite-treated carrots were higher ($P < 0.05$) than the control and 2.5% corn starch treated carrots. The a_L values of 0.05% sulfite-treated and 0.2% sulfite-treated carrots decreased ($P < 0.05$) at the 4-mo period and continually decreased over the 8- and 12-mo periods. At the end of 12-mo, the a_L values of 0.2% sulfite-treated and 2.5% corn starch-treated carrots were higher ($P < 0.05$) than those of controls and 0.05% sulfite-treated carrots. Thus 0.05% sulfite treatment did not have a notable protection effect on the color of dehydrated carrots when stored for a 12 mo.

The b_L value increased ($P < 0.05$) in control carrots from the 8- to 12-mo period (Table 3). The b_L value did not differ ($P > 0.1$) among the control, 0.05% sulfite-treated and 0.2% sulfite-treated carrots at the 12-mo period. The b_L values of the control, 0.05% sulfite-treated, and 0.2% sulfite-treated carrots were higher ($P < 0.05$) than the 2.5% corn starch-treated carrots during all storage periods. The b_L value of 0.2% sulfite-treated carrots did not decrease ($P > 0.1$) over the storage period.

All changes in color pattern of rehydrated carrots during storage were generally the same as dehydrated carrots (Table 4). Reduction of a_L values of rehydrated carrots in the control, 0.05% sulfite-treated, 0.2% sulfite-treated, and 2.5% corn starch-treated was 41, 41, 31, and 15%, respectively, during the 12-mo storage. The 0.05% sulfite treatment did not protect the red color when compared to the control, when stored for 12 mo.

The effects of sulfite and starch were compared on carotene content of dehydrated carrots during storage (Table 5). The highest α-carotene content was found in 2.5% corn starch-treated and 0.2% sulfite-treated carrots ($P < 0.05$). The content of α-carotene decreased as the storage period was extended. The loss of α-carotene in control, 0.05% sulfite-treated, 0.2% sulfite-

Table 4—Effects of sulfite and starch on color of rehydrated carrots during storage

Month	Control	0.05% S	0.2% S	2.5% CS
	L (lightness)			
0	4.0a,(f)	41.5a,(e)	41.1a,(e)	40.5a,(e)
4	42.9a,(e)	40.6b,(ef)	40.1b,(e)	40.1b,(ef)
8	43.9a,(e)	39.2b,(f)	39.9b,(e)	40.1b,(e)
12	43.0a,(e)	41.7a,(e)	40.7ab,(e)	38.9b,(f)
	a_L (redness)			
0	33.4a,(e)	36.0a,(e)	35.7a,(e)	35.3a,(e)
4	25.4c,(f)	29.9b,(f)	33.6a,(f)	32.4a,(f)
8	20.0c,(g)	23.2b,(g)	28.6a,(g)	29.5a,(g)
12	19.8c,(g)	21.1c,(h)	24.6b,(h)	29.9a,(g)
	b_L (yellowness)			
0	24.0a,(f)	24.9a(e)	24.2a,(e)	24.3a,(e)
4	25.1a,(e)	24.0b,(f)	24.0b,(e)	23.9b,(ef)
8	24.1a,(f)	23.1a,(g)	24.0a,(e)	24.0a,(ef)
12	22.7a,(g)	23.1a,(g)	23.7a,(e)	23.2a,(f)

[a] Means represent two replications. S = sodium sulfite and CS = corn starch. Means in the same row with different letters a, b, c, or d and same column for each attribute with different letters (e), (f), (g), or (h) differ ($P < 0.05$).

Table 5—Effects of sulfite and starch on carotene content of dehydrated carrots during storage[a]

Month	Control	0.05% S	0.2% S	2.5% CS
	α-carotene, mg/100 g			
0	51.1c,(e)	47.6d,(e)	55.7b,(e)	61.3a,(e)
4	36.5c,(f)	28.4d,(f)	44.4b,(f)	53.1a,(f)
8	23.5c,(g)	16.8d,(g)	33.2b,(g)	41.6a,(g)
12	13.5c,(h)	12.3d,(h)	15.7b,(h)	26.5a,(h)
	β-carotene, mg/100g			
0	78.0d,(e)	84.1c,(e)	114.2a,(e)	89.3b,(e)
4	54.8b,(f)	50.2c,(f)	84.8a,(f)	82.3a,(f)
8	39.4c,(g)	39.2c,(g)	71.3a,(g)	69.6b,(g)
12	30.8d,(h)	37.1c,(g)	46.6b,(h)	66.0a,(h)
	Total carotene, mg/100g			
0	129.1c,(e)	131.8c,(e)	171.9a,(e)	150.7b,(e)
4	91.4c,(f)	78.7d,(f)	129.3b,(f)	135.5a,(f)
8	63.0c,(g)	56.0d,(g)	104.5b,(g)	111.3a,(g)
12	44.4d,(h)	49.4c,(h)	62.4b,(h)	92.6a,(h)

[a] Means represent two determinations. S = sodium sulfite and CS = corn starch. Means in the same row with different letters a, b, c, or d and same column for each attribute with different letters (e), (f), (g), or (h) differ ($P < 0.05$).

treated, and 2.5% corn starch-treated carrots after 12-mo was 74, 75, 73, and 56%, respectively. The corn starch treatment was more effective in preventing loss of α-carotene than were the control and sulfite treatments.

Initially, the β-carotene content of 0.2% sulfite-treated carrots was higher ($P < 0.05$) than 2.5% corn starch-treated carrots (Table 5). However, β-carotene in 0.2% sulfite-treated carrots decreased ($P < 0.01$) faster than in 2.5% starch-treated carrots over the 4-, 8-, and 12-mo periods. During the 12-mo period, β-carotene loss in the control, 0.05% sulfite-treated, 0.2% sulfite-treated, and 2.5% corn starch-treated carrots was 60, 56, 59, and 26%, respectively.

Similar patterns were observed in total carotene content. Starch treatment prevented the loss of α-, β-, and total carotenes more effectively than did the control and sulfite treatments. Baloch et al. (1987) showed that carrots with a sulfite concentration of 1584 to 9621 ppm and carrots blanched for 3 to 5 min had 129 to 138 mg/100g of carotenoid. After 4 mo storage, carotenoid levels were 67.4–85% of initial values, and 50–62% after 14 mo storage. A high concentration of sulfite effectively protected carotenoid in dehydrated carrots during storage.

In our studies, sulfite ranged from 222–1460 ppm initially. After 4 mo storage, remaining carotenes were 60–75% and sulfite residue contents were 71–1310 ppm. The rates of carotene decrease for 12 mo storage for both sulfite treatments were similar. When sulfite decreased to 18 ppm (in the 0.05% sulfite treated group) to 726 ppm (in the 0.2% sulfite treated group) after 12 mo storage, the total α- and β-carotene contents were ≈36%. However, the carotene content in the 0.2% sulfite treated carrots was higher than that in the 0.05% sulfite treated carrots after 12 mo storage. That was because 0.2% sulfite treated carrots resulted in a higher initial carotene content. Compared to the control carrots (only blanched in water), the 0.05% sulfite treatment did not result in higher carotene content and did not protect against carotene loss during storage.

The method of blanching can cause from 5 to 13% loss of carotenoids (Baloch et al., 1977). β-Carotene was the predominant carotene in all samples and accounted for 60% of total carotenes in dehydrated carrots. The higher carotene content resulted in a higher redness value. Arya et al. (1982) indicated that metabisulfite reduced the formation of browning compounds during dehydration and subsequent storage. Salt and metabisulfite improved the color, texture, and reconstitution of dehydrated carrots.

Arya et al. (1982) reported that carotenoids were relatively stable when water activity (a_w) ranged from 0.32 to 0.57, equivalent to moisture contents from 8 to 12% in freeze-dried carrots. Tomkins et al. (1944), however, reported that the proportion of β-carotene oxidized during storage was higher at 8.2% moisture than at 5.4% moisture. In our study, moisture content did not affect carotene during storage since the moisture contents of all dehydrated carrot samples and stored samples were similar (8% to 9%). Moisture content of the dehydrated carrots did not change during 12-mo storage since samples were hermetically sealed in tin cans.

The beneficial action of sulfite as an antioxidant in stabilizing carotenoids in dehydrated vegetables has been reported (Nutting et al., 1970; Feiberg et al., 1964). Our results indicated that the protective action of sulfite depended mainly on the level of sulfite treatment. Low sulfite, (0.05%, or ≈200 ppm residual sulfite), had limited effect against loss of color and carotene over extended storage. Starch treatment prevented carotene loss and retarded color loss. Starch may be a good substitute for sulfite because of its benefits to both nutrition and health.

CONCLUSIONS

RESULTS INDICATED that corn starch and sulfite can retard loss of redness and total α- and β-carotene breakdown during storage. High sulfite treatment resulted in a higher rehydration ratio than did corn starch treatment. A low concentration of sulfite (0.05% sulfite-treated, 222 ppm residual sulfite) can reduce loss of redness in dehydrated products during storage up to 8 mo. However, such a low concentration of sulfite did not improve retention of β-carotene and total α- and β-carotene during storage. Sensory evaluations are needed to test the effects of low sulfite treatments on quality of dehydrated carrots.

REFERENCES

AOAC, 1990. *Official Methods of Analysis.* Assoc. Official Analytical Chemists, Arlington, VA.

Arya, S.S., Netesan, V., Premaavalli, K.S., and Vijayraghavan, P.K. 1982. Effect of prefreezing on the stability of carotenoid in blanched air dried carrots. J. Food Technol. 7(1): 109–113.

Baloch, A.K., Buckle, K.A., and Edwards, R.A. 1977. Effect of processing variables on quality of dehydrated carrot. II. Leaching losses and stability of carrot during dehydration and storage. J. Food Technol. 12: 295–307.

Baloch, A.K., Buckle, K.A., and Edwards, R.A. 1981. Effect of chemical treatment on stability of dehydrated carrot. Pakistan J. Sci. Ind. Res. 24(5–6): 203–211.

Baloch, A.K., Buckle, K.A., and Edwards, R.A. 1986. Effect of coating with starch and nordihydroguaiaretic acid on the stability of carotenoid of dehydrated carrot. J. Chem. Soc. Pak. 8(1): 59–62.

Baloch, A.K., Buckle, K.A., and Edwards, R.A. 1987. Effect of sulphur dioxide and blanching on the stability of carotenoid of dehydrated carrot. J. Sci. Food Agric. 40: 179–187.

Bhagat, B. and Lockett, M.F. 1964. The effect of sulfite in solid diets on the growth of rats. Food Cosmet. Toxicol. 2: 1–13.

Borchgrevink, N.C. and Charley, H. 1966. Color of cooked carrots related to carotene content. J. Amer. Dietetic Assoc. 49: 116–121.

Bushway, R.J. 1985. Separation of carotenoid in fruits and vegetables by high performance liquid chromatography. J. Liquid Chromat. 8(8): 1527–1547.

DeVries, J.W., Ge, H., Ebert, F.J., Magnuson, J.M., and Ogawa, M.K. 1986. Analysis for total sulfite in foods by using rapid distillation followed by redox titration. J. Assoc. Off. Anal. Chem. 69(5): 827–830.

Eusebio, E.C., Gonzales, J.G., Tobias, J.R., Soriano, L.B., Peralta, E.I., and Marcelo, F.S. 1980. Studies on the dehydration of petsay leaves, kamote tops and carrots. NSDB Technol. J. 5(1): 48–53.

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Low Temperature Blanching Effects on Chemistry, Firmness and Structure of Canned Green Beans and Carrots

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ABSTRACT

Green beans and carrots were canned using extended blanching at 64–65°C and added calcium and/or acid. Firmer products resulted from all treatments but lowered pH was most effective. Blanched green beans were firmer with lower pectin esterification, indicating pectin methyl esterase activity. Green beans and carrots treated with calcium and/or acid and then cooked were firmer than controls. Acid exhibited a firming effect, perhaps by loosening tissue, while calcium reduced the influence of heat. Instrumental bioyield values correlated with sensory results of canned green beans; bioyield may result from a sclerifomic layer. Microscopy showed firmer beans had intact middle lamellae while softer samples contained separated cells. These data suggest that the treatments rendered pectates in the middle lamella less heat labile.

Key Words: carrots, green beans, low temperature, blanching, firmness

INTRODUCTION

FRUIT AND VEGETABLE MARKETS show increased demand for 'fresh-like' products, motivated largely by preferences for firmer textures than found in canned products and enhanced health awareness. Such foods are termed 'minimally processed' or 'partially processed' and derive their 'fresh-like' quality from the short and mild heat treatments that are used. However, such products are not commercially sterile and there are major needs for extending their shelf life (Rolle and Chism, 1987; Shewfelt, 1987; Labuza and Breene, 1989; Watada et al., 1990).

Canning, while providing the advantage of long shelf life (typically several years), results in vegetables with less desired textural qualities since the heat treatment necessary to ensure safety produces extensive pectin hydrolysis and loss of firmness. Technology to eliminate the softening associated with canning may cause the trend away from canned vegetables to be reversed. Bourne (1989) reported that blanching vegetables at 60–65°C before canning resulted in significantly firmer products. This low temperature blanch also produced a stronger response to added calcium as a firming agent and to pH adjustment ≤4.5 that enabled a gentler heat process to achieve commercial sterility.

Addition of acid (Townsend et al., 1954) or calcium (Kertesz et al., 1940) to processed plant tissue induces firming and both treatments are permitted by the U.S. Food & Drug Administration. For example, added calcium is a firming agent for both green beans (Van Buren et al., 1988) and carrots (Sterling, 1968), among others. The mechanism of this reaction has been considered to involve divalent cation crosslinking between adjacent pectin molecules through reactive carboxyl groups (Van Buren, 1979). Pectin methylesterase (PME) can hydrolyze methyl groups from pectin chains, increasing the number of carboxyl groups available to react with divalent cations. This enzyme is endogenous to many plant tissues and allowing this reaction to proceed by incubation at elevated temperatures may produce a firming action in vegetables (Bartolome and Hoff, 1972).

The U.S. Food & Drug Administration is amending the standards of identity for canned green beans to allow use of glucono-δ-lactone to lower pH and reduce processing time and temperature (Anon., 1992). The mechanism for reduced pH to achieve firmness is less understood than that of calcium. The texture of plant tissue must relate to its structure and mechanical strength. As a first approximation, the strength of the cell walls would seem to influence texture (Sterling, 1968). Pitt (1982) described three components of cellular plant tissue that determined textural response. These include fluid-filled, thin-walled parenchyma cells, the pectinaceous layer between adjacent cells or middle lamella, and the extracellular volume which may either contain fluids or interstitial air.

Heating produces structural alterations in plant tissue that influence texture (Aguilera and Stanley, 1990). The general result is softening, brought about by loss of turgor pressure and occluded air, thermal degradation of middle lamella pectins and other cell wall polysaccharides, and starch gelatinization. Structurally, the most obvious result of heating is cell separation as a result of thermal destabilization of pectic materials.

Practically, pectic materials in fruits and vegetables may be considered as calcium salts of polymers of galacturonic acid that have been partially esterified with methyl alcohol (Kertesz et al., 1940; Bourne, 1976). The degree of polymerization and esterification of the polygalacturonide chains and the amount of salt formation have important effects on the physical properties of the middle lamella and resulting texture. Pectin chains are hypothesized to bond via hydrogen bonding and interaction of methyl groups; also, the chains carry negative charges and the charge density is greater at high pH, due to the dissociation of carboxyl groups leading to chain repulsion and lowered interchain association (Van Buren et al., 1988).

Sterling (1968) reported values for textures of cooked carrot tissue prepared by boiling core or xylem tissue in buffers ranging from pH 3.0 to 8.0 and tested for firmness in a texturometer. Firmness values decreased linearly with increasing pH (R^2 = 0.847). Concomitant light microscope (LM) examination of cross-sections showed that at low pH the parenchymous cells were tightly bound to one another, while at higher pH cell separation occurred as well as cell collapse. He reported similar effects of pH on pectin solubility and strength of pectin gels. Transmission electron microscope (TEM) studies of carrot tissue (Ahmed et al., 1991) showed that cell walls became less dense as a result of heating. Van Buren et al. (1988) found that increasing pH in the range 4.0–7.0 led to decreased firmness of green beans but the degree of this effect could be diminished by increasing ionic strength with the monovalent cation, sodium. This was hypothesized to result from providing sufficient counterions to reduce the charge density on pectin chains containing dissociated carboxyl groups so that there would be little effect of the degree of dissociation controlled by pH. Also, the effect of pH could be negated by the addition of excess Ca^{+2}. This implies that pH influences the binding of Ca^{+2} by pectin, as would be expected since calcium salts are less soluble at higher pH.

Similar results were obtained by McFeeters and Fleming (1991) for cucumber tissue. They found that Ca^{+2} had little in-

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Table 1—Canning treatments for green beans and carrots

Green beans					Sample #				
Treatment #	61	62	63	64	65	66	67	68	
Hold time (min, 65°C)	0[a]	0[b]	30	30	30	30	60	60	
Brine[c] additions	—	—	—	C[d]	A[e]	C+A	—	C	
Retort temp. (°C)	121	121	121	121	102	102	121	121	
Retort time (min)	13	13	13	13	13	13	13	13	
Carrots					**Sample #**				
Treatment #	41	42	43	44	45	46	47	48	49
Hold time (min, 64°C)	8[a]	30	30	30	30	60	60	60	60
Brine[c] additions	—	—	C	A	C+A	—	C	A	C+A
Retort temp. (°C)	121	121	121	102	102	121	121	102	102
Retort time (min)	23	23	23	15	15	23	23	15	15

[a] Blanch temperature - 97°C.
[b] Blanch temperature - 79°C.
[c] 1.75% NaCl.
[d] 0.27% $CaCl_2$.
[e] 0.28% citric acid + 4% sugar.

Table 2—Firmness, pH and calcium values for canned green beans and carrots

	Green beans				Carrots		
Sample #	Firmness (N)[a]	pH[b]	Ca^{+2c}	Sample #	Firmness (N)[a]	pH[b]	Ca^{+2c}
61	212 (6.3)	5.07	0.81	41	147 (6.0)	4.88	0.58
62	276 (12.4)	5.07	0.71	42	224 (10.7)	4.96	0.62
63	381 (10.1)	5.06	0.63	43	350 (20.2)	4.84	1.44
64	620 (36.4)	5.01	1.69	44	2819 (232)	4.10	0.55
65	3564 (124)	4.01	0.54	45	3494 (241)	4.04	1.01
66	3858 (85.8)	3.91	1.29	46	249 (18.4)	4.98	0.65
67	418 (12.6)	5.07	0.78	47	419 (25.9)	4.86	1.39
68	668 (24.8)	4.95	1.84	48	2958 (185)	4.15	0.49
				49	3540 (190)	4.09	1.07

[a] Results from extrusion cell apparatus; average of at least five determinations (std. dev.).
[b] Average of two determinations.
[c] Average of two determinations; results expressed as % (db).

Table 3—ANOVA results of canned green bean and carrot firmness data[a]

Green beans	R^2 = 0.9977, Root Mean Square Error = 0.0481		
Source	df	F Ratio	Prob > F
Acid (A)	1	.6222	0.5748
Calcium (C)	1	1.2971	0.4587
Enzyme (E)	1	0.7884	0.5378
A × C	1	0.8174	0.5320
A × E	1	0.7380	0.5482
C × E	1	2.5670	0.3552
Carrots	R^2 = 0.9445, Root Mean Square Error = 0.1546		
Source	DF	F Ratio	Prob > F
Acid (A)	1	0.7154	0.4867
Calcium (C)	1	4.8037	0.1597
Holding time (T)	1	3.4070	0.2062
A × C	1	3.6178	0.1975
A × T	1	4.3178	0.1733
C × T	1	0.5737	0.5279

[a] Data used include probe puncture estimations of firmness for reheated acid treatments, pH values, calcium levels and enzyme activity (beans) or holding times (carrots). Variables were treated as continuous.

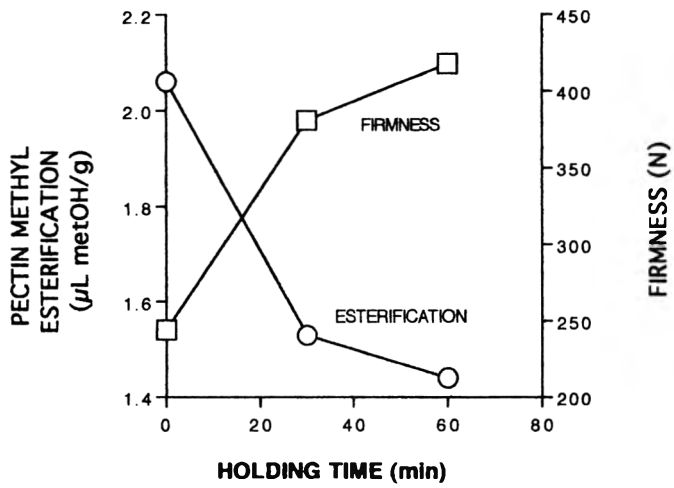

Fig. 1—Relationship of degree of pectin methyl esterification (○--○) to firmness (□--□) in three green bean samples held at 65°C for various times prior to thermal processing.

hibitory effect on the rate of softening at pH > 5.0 but the effectiveness increased as pH was lowered. They questioned the importance of β-elimination and acid hydrolysis of pectin in softening of tissue and suggested that conformational changes of this molecule may be important.

Our objectives were to determine if the addition of calcium and/or acid and the activation of endogenous PME would produce a firm canned product and to study the effects of calcium and pH in firming green bean and carrot tissue during heating.

MATERIALS & METHODS

Vegetables

For canning experiments, fresh green beans (Acclaim cultivar) and carrots (PY 60 cultivar) grown locally were obtained from a Geneva, NY, cannery. These were washed and trimmed prior to being cut into ≈4 cm lengths (green beans) or sliced ≈5 mm thick (carrots). For soaking and cooking experiments, fresh green beans and carrots picked locally were obtained from a Guelph, Ontario, vegetable retailer. These were washed and trimmed prior to being cut into ≈5 cm lengths (green beans) or sliced ≈3 mm thick (carrots).

Canning

Green beans in 8 kg batches were blanched either in hot water (79 or 97°C) for 3 min (commercially blanched controls), or in hot water (65°C) for 3 min, placed in a covered container and held for various times (low temperature blanched treatments). The beans were then packed into #303

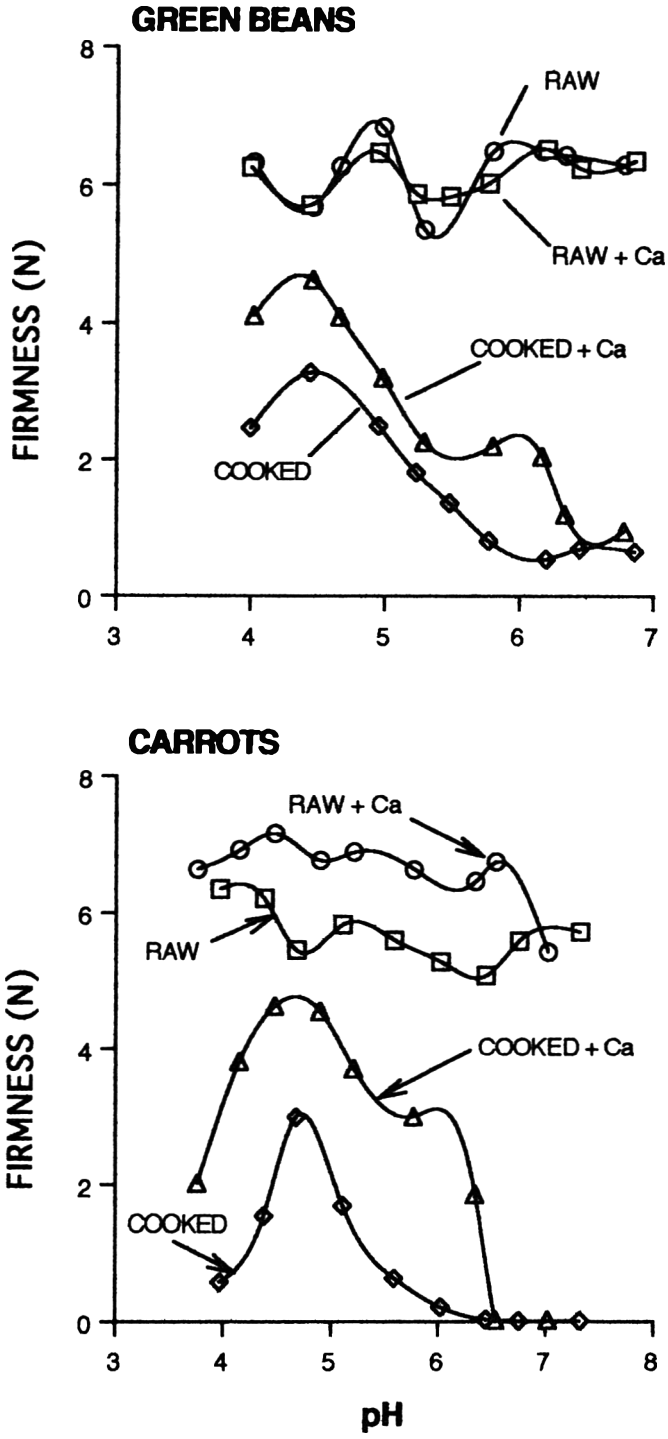

Fig. 2—Influence of pH and calcium on firmness of raw and cooked green beans and carrots.

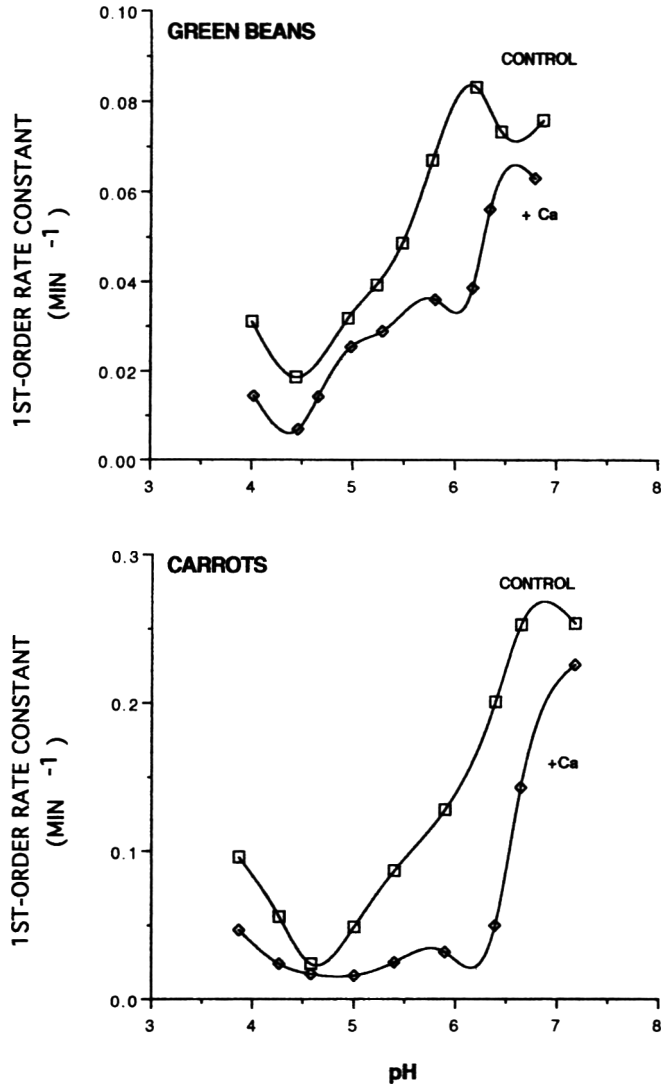

Fig. 3—Influence of pH and calcium on first-order rate constants for cooking of green beans and carrots.

cans. covered with hot brine with or without additives, sealed, retorted and then water cooled (Table 1). Cans were held at room temperature (≈23°C) prior to testing. Carrots were treated in the same fashion, except that they were blanched whole in water in a steam-jacketed kettle at 97°C for 8 min (commercially blanched control), or held in water at 64°C for various times (low temperature blanched treatments). The carrots were then peeled using near-boiling 10% NaOH, hand trimmed and machine sliced. Note that for both green beans and carrots, any treatment that included acid was retorted at a lower temperature and, in the case of carrots, for a shorter time. To compensate for this, several cans of acid-containing bean treatments were further retorted at 121°C for 13 min while the acid-containing carrot treatments received an additional 23 min heating.

Soaking and cooking

Fresh cut green beans and sliced carrots were blanched in boiling water for 3 min, quenched in ice water, and ≈50 g samples weighed into beakers containing 100 mL buffer solution consisting of 0.05M phosphate/citrate buffer ± 0.024M $CaCl_2$, ranging in nominal pH from 3.0 to 7.0 in 0.5 pH unit steps. Note that at nominal pH values ≥ 6.0 added calcium formed a complex with the buffer salts. The beakers were covered and stored for 36–40 hr at 5°C. pH values were then determined by glass electrode from a homogenate prepared with ≈10 g rinsed material and 100 mL distilled water. The remaining sample was cooked for 30 min (green beans) or 25 min (carrots) on a hot plate. Beakers were covered with watch glasses to eliminate evaporation. Samples for firmness determinations were taken of the raw material and after cooking.

Another series of experiments was performed in which cooking times were varied at different pH levels. Green bean samples were soaked at pH 4.0, 5.5 and 7.0, with and without added calcium, and cooked for 0, 5, 10, 20 and 30 min. Carrot samples were soaked at pH 4.5, 5.5 and 6.5, with and without added calcium, and cooked for 0, 5, 10 and 20 min.

Firmness

Green bean and carrot samples from canning experiments were evaluated for firmness using a back extrusion cell (Bourne and Moyer, 1968). Samples were taken from 3 cans and 5 (green bean) or 7–9 (carrot) samples tested/treatment. Firmness was expressed as force (N).

Green bean and carrot samples from soaking and cooking experiments as well as canned samples were puncture tested at a deformation speed of 10 mm min^{-1} using a 1.6 mm diameter flat-ended cylindrical probe attached to a "M" series materials testing machine (J. J. Lloyd Instruments, Hamilton, Ont.) equipped with a 50N load cell. Green beans were split along the median line and one half of each pod centered outer or

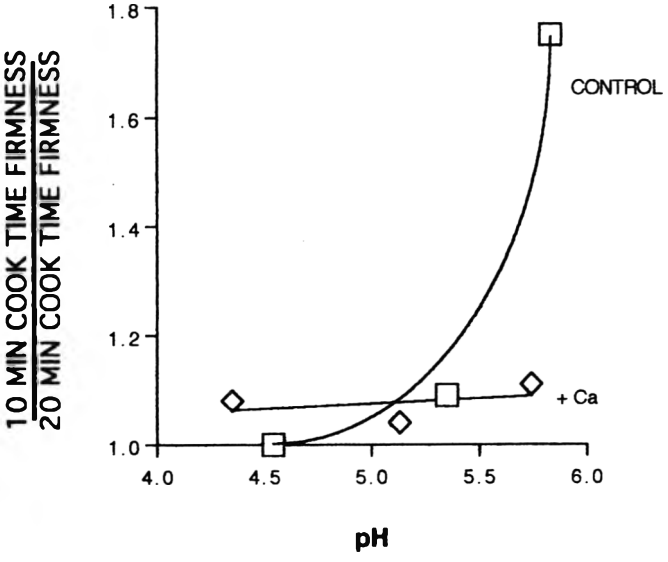

Fig. 4—Influence of pH and calcium on the ratio of 10 min to 20 min cook time firmness in green beans.

epidermal side up on a steel jig over an 8 mm hole so that the probe entered the specimen at right angles to the long axis of the pod. For canned material, samples were taken from two cans and four samples tested/can. For material from soaking and cooking experiments, one measurement was obtained per sample and four samples were taken/treatment; each treatment was duplicated. Carrot slices were centered over the jig hole and tested in the core or xylem tissue so that the probe entered the specimen parallel to the long axis of the root. One measurement was obtained/sample and six samples were taken/treatment; each treatment was duplicated. Force-deformation curves were recorded and stored electronically; computer analysis resulted in F_{max} (N) and deformation values (mm) for firmness.

Preparation of cell wall material and analysis of cell wall pectin

Samples of green beans were taken following the blanching period, frozen and stored at −20°C in sealed polyethylene bags prior to analysis. Cell walls were prepared according to the method of Rushing and Huber (1990) as modified by Jackman et al. (1992). Isolated cell walls were frozen, lyophilized and stored in a desiccator until analyzed. These samples were assayed in duplicate for total cell wall uronic acid content (i.e., pectin) as described by Ahmed and Labavitch (1977) using galacturonic acid as the standard. The degree of cell wall pectin methyl esterification was measured by the method of Wood and Siddiqui (1971). All reported values were corrected for both reagent and sample blanks. Results were expressed as mL methanol/g cell wall material (db).

Chemical analysis

Samples of green beans and carrots from all canning treatments were analyzed for calcium using atomic absorption methodology following digestion by acid and heat (Thomas et al., 1967). Two samples, consisting of five drained and rinsed pieces from different cans, were analyzed and the results expressed as % (db).

Microscopic analysis

Samples of green beans from all canning treatments were prepared for microscopic analysis by glutaraldehyde fixation, critical-point drying and sputter coating with gold to a thickness of 300 Å. Samples were examined in a Hitachi S-570 scanning electron microscope (SEM) (Hitachi Ltd., Tokyo, Japan) operated at 10 kV.

Sensory analysis

Samples of green beans from all canning treatments were evaluated by a sensory analysis group consisting of 17 male and female graduate and undergraduate students who were given a training session prior to the formal panel. The group was asked to evaluate only the texture and color of coded samples tested at room temperature (≈23°C). In order to evaluate texture, judges first estimated the force needed to puncture the outside bean surface using the large end of a hand-held flat wooden toothpick. These data ("Hand") were recorded by placing a mark on a 10 cm horizontal line anchored at the left by "little force" and on the right by "much force." Following this, judges estimated the force needed for the front teeth to bite through a bean using a scale similar to that used previously to record the data ("Tooth"). Finally, judges estimated color of the beans on a 10 cm horizontal line anchored at the left by "light color" and on the right by "dark color". As well as the experimental treatments, a control sample of commercial "No Name" product was used. Data analysis consisted of measuring the distance in cm from the left anchor; the value for the commercial control was set to zero and all other responses, positive or negative, were measured from this point. Samples were evaluated on two separate days but not all panelists participated in both sessions.

Statistical analysis

Instrumental texture data were stored and graphed using Cricket Graph (Cricket Software, Malvern, PA), results were tabulated using Lotus 1-2-3 for Macintosh (Lotus Development Corp., Cambridge, MA) and statistical analysis performed using JMP for Macintosh (SAS Institute Inc., Cary, NC).

RESULTS & DISCUSSION

THE CANNING TREATMENTS influenced the firmness, pH and calcium levels of the resulting green bean and carrot tissue (Table 2). In order to assess the effectiveness of the treatments in retaining firmness, ANOVA was performed using probe puncture values and results from the reheated acid treatments. Results (Table 3) indicated that while all main effects increased firmness, none did so significantly in isolation. Interactions were not significant. However, the R^2 values of the two complete models both exceeded 90%, indicating a high proportion of the variation in firmness could be explained by treatments and interactions.

To determine whether the added firmness in samples undergoing an extended hold time resulted from endogenous PME activity, cell wall material from frozen green bean tissue was assayed for pectin methyl esterification. Samples 61 and 62 had been blanched (although at different temperatures) for 3 min and then cooled immediately, sample 63 had no added calcium or acid and was held for 30 min at the lower blanch temperature of 65°C, while sample 67 had no added calcium or acid and was held for 60 min. Thus, chemical and firmness data from samples 61 and 62 were averaged and compared to results from samples 63 and 67. This resulted in three valid points for comparison. When these data were plotted (Fig. 1) increasing holding time obviously promoted enzyme activity, as evidenced by lower esterification values, and higher values for firmness, presumably resulting from crosslinking through endogenous calcium. Further, there was a high correlation (R^2 = 0.995) between firmness and degree of pectin methyl esterification. Although only a small number of samples were tested, these data suggest a firming mechanism based on endogenous PME activity. Also, prior work (Bartolome and Hoff, 1972), has proposed a mechanism for heat-induced firming based on PME activity. The relationship between pectin methylation and tissue firmness is not simple. Hudson and Buescher (1986) reported firmer tissue as degree of pectin esterification increased in cucumbers; calcium treatment reduced esterification and prevented unwanted softening. They considered this effect a result of conformational changes in demethylated pectin that loosened middle lamella components.

Enhancing PME activity through extending holding times had a firming effect on both beans and carrots, as did the addition of acid and calcium. To study the acid/calcium effects in more detail, the soaking and cooking experiments were carried out. Lowering pH values and adding calcium generally resulted in greater firmness down to pH 4.5 for both cooked samples, while for raw samples the only significant effect was that of added calcium in carrots (Fig. 2). Below pH 4.5 firmness decreased, perhaps as a result of acid hydrolysis.

Table 4—Sensory and instrumental data for canned green beans

Sample #	Sensory (cm)			Instrumental (N)		
	Hand	Tooth	Color	Extrusion	Puncture	Bioyield
Commercial	0.0	0.0	0.0			
61	0.0 (0.83)	0.0 (0.87)	2.4 (1.0)	212 (6.3)	0.38 (0.06)	0.28 (0.05)
62	0.8 (0.49)	0.6 (0.68)	3.1 (0.46)	276 (12.4)	0.47 (0.08)	0.46 (0.11)
63	1.6 (0.65)	1.6 (0.72)	3.8 (0.46)	381 (10.1)	0.62 (0.12)	0.51 (0.03)
64	4.0 (0.92)	3.3 (0.75)	4.7 (0.35)	620 (36.4)	0.93 (0.15)	0.88 (0.16)
65	8.0 (0.83)	8.1 (0.84)	5.9 (1.2)	3564 (124)	4.74 (1.0) 1.13 (0.19)[a]	1.80 (0.05)
66	8.4 (0.86)	8.4 (0.69)	7.0 (0.97)	3858 (86)	4.94 (0.80) 1.52 (0.21)[a]	1.81 (0.06)
67	3.3 (0.93)	3.2 (0.92)	6.7 (0.84)	418 (12.6)	0.66 (0.09)	0.52 (0.10)
68	4.3 (0.72)	3.6 (0.90)	5.9 (0.71)	688 (24.8)	1.01 (0.20)	0.83 (0.09)

[a] Puncture values for reheated acid-containing treatments.

Table 5—Correlation coefficients for sensory and instrumental data of canned green beans[a]

Parameter	Hand	Tooth	Color	Extrusion	Puncture	Bioyield
Hand	1.000					
Tooth	0.995	1.000				
Color	0.828	0.811	1.000			
Extrusion	0.917	0.944	0.610	1.000		
Puncture	0.920	0.947	0.611	1.000	1.000	
Bioyield	0.969	0.975	0.674	0.972	0.974	1.000

[a] df. Significant values for r at 6 df: $P \leq 0.05 = 0.707$; $P \leq 0.01 = 0.834$.

We determined in separate experiments that when log of puncture force was plotted vs cook time a straight line resulted (data not shown). Thus cooking under these conditions followed apparent first-order kinetics and a rate constant could be calculated (Labuza and Kamman, 1983; Rao and Lund, 1986). Results for cooked green beans and carrots (Fig. 3) were expressed as apparent first-order rate constants vs pH (McFeeters and Fleming, 1991) and, except for the numerical value of the constant, quite similar trends were found in both cases. Added calcium resulted in lowered cooking rate constants over the entire pH range while declining pH values produced a generally linear decrease in this parameter in the range of pH 6.5 to 4.5 whereupon rate constants increased. Calcium had more effect at higher pH values (up to pH ~ 6.0–6.5; above that the insolubility of calcium may have become a factor) which may explain the interaction of calcium and enzyme level or holding time in heat processed green beans (Table 3). The lower level of endogenous calcium may reflect a limiting role for this ion in crosslinking reactions, so that added calcium would be necessary for this reaction to proceed.

When effects of pH levels and added calcium were compared for raw, cooked and retorted treatments we observed that, although for raw material calcium produced some firming in carrots compared to a buffer control, the effect of pH was negligible. In cooked samples, soaking in calcium led to definite firming compared to buffer controls. For retorted samples calcium in the brine produced a more firming effect compared to buffer control, although the reduced thermal process in samples containing acid must be considered. These findings led to the initial conclusion that as heat treatment increased the tissue became more disrupted, leading to greater infusion of buffer and stronger effects from both pH and calcium. The general influence of heating on plant material is to loosen the cellular structure (Aguilera and Stanley, 1990). Sterling (1968) reported a differential effect of pH on structure during cooking of carrots. The more acidic treatments exhibited less cell separation and collapse than those at higher pH.

In order to test the influence of heat treatment, a series of experiments was performed with fresh green beans cooked for 10 or 20 min at different pH levels. Increased heating (Fig. 4) greatly facilitated the effects of acidic pH to retard thermal softening, while calcium plus acid became more effective than acid

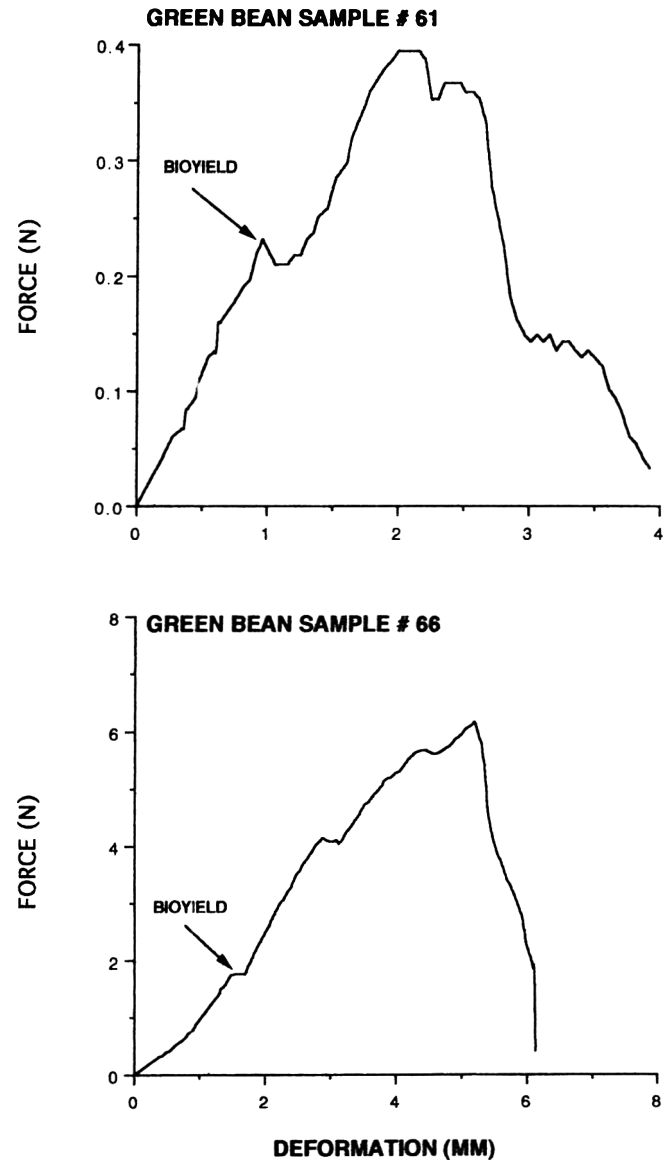

Fig. 5—Force-deformation curves of puncture tests for canned green bean samples 61 and 66 (unreheated) showing bioyield.

alone only at higher pH and was only slightly influenced by heat. Thus, under these conditions heat enhanced the effects of pH in achieving firmness but had less effect if calcium was also present. This was perhaps due to the cross-linking ability of this divalent cation on pectic substances.

Sensory analysis on the canned beans samples verified instrumental data. The results (Table 4) and corresponding correlation matrix (Table 5) showed that all three instrumental methods

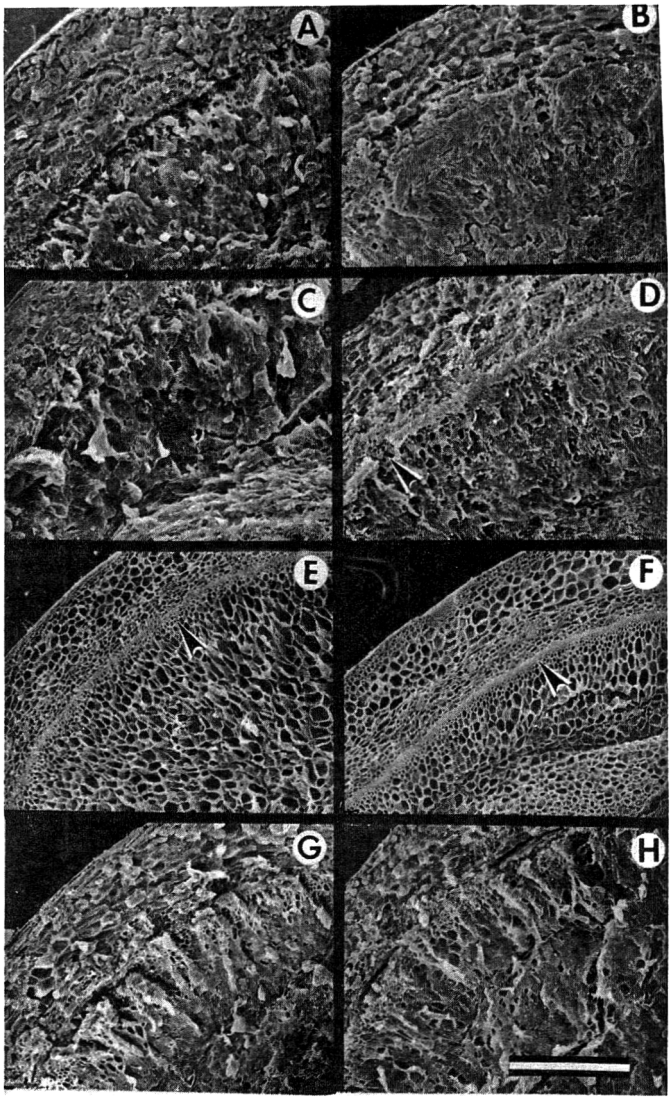

Fig. 6—SEM micrographs of canned green beans. Sample numbers: (A) 61; (B) 62; (C) 63; (D) 64; (E) 65 (unreheated); (F) 66 (unreheated); (G) 67; (H) 68. Bar = 0.50 mm. Arrows designate the fiber sheath.

were strong predictors of sensory texture response ($R^2 \geq 0.84$). Color positively correlated with sensory and instrumental texture; however, a 'halo' effect, in which the judgment of one parameter influences the judgment of another must be considered. All experimental treatments produced color values higher than the commercial sample ($P \leq 0.01$), although a difference in cultivar may be important. Similar reductions were seen for these treatments with carrots (data not shown).

Of the three instrumental values for firmness (Table 4), force at the bioyield provided the strongest correlation with both sensory responses (Table 5). This parameter was defined as the first small initial peak in the force—deformation puncture curve (Fig. 5). It appeared in over half the samples tested but was found rarely when the beans were tested inner side up, and then only in the firmest tissue. The force-deformation curves were almost linear up to the bioyield point and, following a small region of pseudoplastic deformation, continued in this fashion to failure. Following failure, a second but smaller peak often occurred. Similar curves with bioyield points have been noted for green and ripe tomato tissue; Jackman and Stanley (1992) reported a bioyield of ~7 N for puncture testing of mature-green tomatoes and this value did not decrease with ripening. A bioyield point has also been observed for other fruits and vegetables (Mohsenin et al., 1963; Fletcher et al., 1965). Jackman and Stanley (1992) attributed bioyield in tomato pericarp to some property of the tissue rather than to the testing procedure since bioyielding was found consistently in both punctured and flat plate compressed samples at deformation rates of 0.5–50 mm min^{-1}. Although the cause of bioyield in tomato pericarp was not identified precisely we hypothesized that it could have resulted from compaction of interstitial air spaces or plastic deformation of the middle lamella which would allow for cell rearrangement during compression.

To better understand the significance and cause of bioyield, samples of canned green bean treatments were examined using the SEM. These data (Fig. 6) showed in firmer samples the appearance of a radially located layer of small compressed cells close to the bean surface. These appeared to be the scleriformic layer termed the fiber sheath (Reeve and Brown, 1968 a,b) which is composed of partially lignified, thick-walled sclereid cells. As the bean passes through the stages of edible maturity, wall thickening and lignification increases (Reeve and Brown, 1968b). This could explain the variation in bioyield found in the canned bean samples which likely represented a spectrum of maturities, and, hence, a range of initial firmness. Because of their compressed nature, these cells would be more resistant to puncture force than the surrounding tissue and this may account for the appearance of a bioyield. Since it may be the firmest tissue encountered during puncture it could have a strong effect on sensory responses. Attempts to measure the deformation at which bioyield occurred from force-deformation curves resulted in a value of 1.66 mm ± 0.29, while estimating the depth of these cells from the bean surface using SEM micrographs yielded 0.72 mm ± 0.12. These types of measurements involve the tendency for force measuring instruments to overestimate, and the underestimation of SEM measurements due to angle. Thus we could not assume that these two measurements were different. Studies of other species will be necessary before this mechanism for bioyield can be confirmed.

Another feature of the micrographs (Fig. 6) is the striking contrast between more firm and less firm tissue. Treatments resulting in higher firmness for canned beans (e.g., 65, 66, not reheated) exhibited a microstructure composed of cells joined by a functioning middle lamella. Those treatments resulting in lower firmness values (e.g., 61, 62) had an appearance characteristic of plant material in which the middle lamella had been thermally degraded, resulting in separated cells. It was possible to discern portions of the intact fiber sheath layer in the less firm samples (e.g., 64, Figure 6) when the cells were not obscured by separated parenchyma. The fiber sheath would be expected to be heat resistant due to lignification. These data correspond to those of Sterling (1968) and Ahmed et al. (1991), among others, and suggested strongly that the mechanism of firming of the treatments was to prevent thermal degradation of the middle lamella.

CONCLUSIONS

These treatments were effective and a range of firmness could be produced, depending upon treatment level. Increasing the holding time of green bean samples at 65°C resulted in firmer tissue and increasing firmness values correlated with decreasing pectin methyl esterification. This would enable more pectin molecules to enter into cross-linking reactions with calcium. Increasing acid levels were associated with firmer tissue in cooked samples. Acid, if pH values were < 4.5, also enables processing at a much lower temperature, which greatly decreases thermal degradation. Calcium enhances this effect, particularly at higher pH values. As cooking proceeds, acid has more firming effect, perhaps by loosening the tissue to allow greater penetration, while the addition of calcium to acid brine reduced the influence of heat. The methods increased firmness of canned green beans and carrots and were effective especially in combination. Consumer acceptance responses to these products are needed to determine optimum firmness. With higher pH, the addition of calcium can also contribute to firmness.

REFERENCES

Aguilera, J.M. and Stanley, D.W. 1990. *Microstructural Principles of Food Processing and Engineering*. Elsevier Applied Science, London.

Ahmed, A. and Labavitch, J.M. 1977. A simplified method for accurate determination of cell wall uronide content. J. Food Biochem. 1: 361–365.

Ahmed, E.M., Mirza, S., and Arreola, A.G. 1991. Ultrastructural and textural changes in processed carrot tissue. J. Food Qual. 14: 321–330.

Anon. 1992. Washington News. Food Technol. 46(10): 58.

Bartolome, L.G. and Hoff, J.E. 1972. Firming of potatoes: biochemical effects of preheating. J. Agric. Food Chem. 20: 266–270.

Bourne, M.C. 1976. Texture of fruits and vegetables. In *Rheology and Texture in Food Quality* J.M. deMan, P.W. Voisey, V.F. Rasper, and D.W. Stanley (Ed.), p. 275–307. AVI Publishing Company, Westport, CT.

Bourne, M.C. 1989. How kinetic studies of detergency with Walter Jennings led to firmer textured processed vegetables and fruits. 198th American Chemical Society National Meeting. Miami, Sept. 12. Division of Agriculture & Food Chemistry Abstract No. 28.

Bourne, M.C. and Moyer, J.C. 1968. The extrusion principle in texture measurement of fresh peas. Food Technol 22: 1013–1018.

Fletcher, S.W. III, Mohsenin, N.N., Hammerle, J.R., and Tukey, L.D. 1965. Mechanical behavior of selected fruits and vegetables under fast rates of loading. Trans. Am. Soc. Agric. Eng. 8: 324–326, 331.

Hudson, J.M. and Buescher, R.W. 1986. Relationship between degree of pectin methylation and tissue firmness of cucumber pickles. J. Food Sci. 51: 138–140, 149.

Jackman, R.L. and Stanley, D.W. 1992. Area- and perimeter-dependent properties and failure of mature-green and red-ripe tomato pericarp tissue. J. Texture Studies 23: 461–474.

Jackman, R.L., Gibson, H.J., and Stanley, D.W. 1992. Effects of chilling on tomato fruit texture. Physiol. Plant. 86: 600–608.

Kertesz, Z.I., Tolman, T.G., Loconti, J.D., and Ruyle, E.H. 1940. The use of calcium in the commercial canning of whole tomatoes. NYS Agr. Exp. Sta. Tech. Bull. No. 252.

Labuza, T.P. and Kamman, J.F. 1983. Reaction kinetics and accelerated tests simulation as a function of temperature. In *Computer-Aided Techniques in Food Technology*, I. Saguy (Ed.), p. 71–115. Marcel Dekker, Inc., New York.

Labuza, T.P. and Breene, W.M. 1989. Applications of "active packaging" for improvement of shelf life and nutritional quality of fresh and extended shelf-life foods. J. Food Proc. Preserv. 13: 1–69.

McFeeters, R.F. and Fleming, H.P. 1991. pH effect on calcium inhibition of softening of cucumber mesocarp tissue. J. Food Sci. 56: 730–732, 735.

Mohsenin, W.N., Cooper, H.E., and Tukey, L.D. 1963. Engineering approach to evaluating textural factors in fruits and vegetables. Trans. Am. Soc. Agr. Eng. 6: 85–88, 92.

Pitt, R.E. 1982. Models for the rheology and statistical strength of uniformly stressed vegetative tissue. Trans. ASAE 25: 1776–1784.

Rao, M.A. and Lund, D.B. 1986. Kinetics of thermal softening of foods—a review. J. Food Process. Preserv. 10: 311–329.

Reeve, R.M. and Brown, M.S. 1968a. Histological development of the green bean pod as related to culinary texture. 1. Early stages of pod development. J. Food Sci. 33: 321–326.

Reeve, R.M. and Brown, M.S. 1968b. Histological development of the green bean pod as related to culinary texture. 2. Structure and composition at edible maturity. J. Food Sci. 33: 326–331.

Rolle, R.S. and Chism III, G.W. 1987. Physiological consequences of minimally processed fruits and vegetables. J. Food Qual. 10: 157–177.

Rushing, J.W. and Huber, D.J. 1990. Mobility limitations of bound polygalacturonase in isolated cell wall from tomato pericarp tissue. J. Amer. Soc. Hort. Sci. 115: 97–101.

Shewfelt, R.L. 1987. Quality of minimally processed fruits and vegetables. J. Food Qual. 10: 143–156.

Sterling, C. 1968. Effects of solutes and pH on the structure and firmness of cooked carrot. J. Fd. Technol. 3: 367–371.

Thomas, R.L., Sheard, R.W., and Moyer, J.R. 1967. Comparison of conventional and automated procedures for N, P, and K analysis of plant material using a single digestion. Agron. J. 59: 240–243.

Townsend, C., Yee, L., and Mercer, W.A. 1954. Inhibition of the growth of *Clostridium botulinum* by acidification. Food Res. 19: 536–542.

Van Buren, J.P. 1979. The chemistry of texture in fruits and vegetables. J. Texture Studies 10: 1–23.

Van Buren, J.P., Kean, W.P., and Wilkison, M. 1988. Influence of salts and pH on the firmness of cooked snap beans in relation to the properties of pectin. J. Texture Studies 19: 15–25.

Watada, A.E., Abe, K., and Yamuchi, N. 1990. Physiological activities of partially processed fruits and vegetables. Food Technol. 44(5): 116–122.

Wood, P.J. and Siddiqui, I.R. 1971. Determination of methanol and its application to measurement of pectin ester content and pectin methyl esterase activity. Anal. Biochem. 39: 418–428.

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Bolin, H.R. and Huxsoll, C.C. 1991. Control of minimally processed carrot (*Daucus carota*) surface discoloration caused by abrasion peeling. J. Food Sci. 56: 416–418.

Bolin, H.R. 1992. Retardation of surface lignification on fresh peeled carrots. J. Food Proc. Pres. 16: 99–103.

Buick, R. and Damoglou, A. 1987. The effect of vacuum packaging on the microbial spoilage and shelf-life of "ready-to-use" sliced carrots. J. Sci. Food Agric. 38: 167–175.

Carlin, F., Nguyen-The, C., Hilbert, G., and Chambroy, Y. 1990. Modified atmosphere packaging of fresh, ready-to-use grated carrots in polymeric films. J. Food Sci. 55: 1033–1038.

Gardner Laboratory, Inc. 1975. Color and color related properties. A1, Appearance measurements, p. 1–3. Gardner Laboratory, Inc., Bethesda, MD.

Howard, L.R. and Griffin, L.E. 1993. Lignin formation and surface discoloration of minimally processed carrot sticks. J. Food Sci. 58: 1065–1067, 1072.

Howard, L.R., Griffin, L.E., and Lee, Y. 1994. Steam treatment of minimally processed carrot sticks to control surface discoloration. J. Food Sci. 59: 356–358, 370.

Judd, D.B. 1963. *Color in business, science and industry*, p. 299–300. John Wiley. New York.

Laschimke, R. 1989. Investigation of the wetting behaviour of natural lignin—a contribution to the cohesion theory of water transport in plants. Thermochimica Acta. 151: 35–56.

O'Rear, L. and Flore, J. 1983. Quantitative and qualitative characterization of carrot root periderm during development. J. Amer. Soc. Hort. Sci. 108: 923–928.

Rooke, E.A. and Van den Berg, L. 1985. Equilibrium relative humidity of plant tissue. Can. Inst. Food Sci. Technol. J. 18: 85–88.

Saguy, I. and Mannheim, C.H. 1975. The effect of selected plastic films and chemical dips on the shelflife Marmade tomatoes. J. Food Technol. 10: 547–556.

Shirazi, A. and Cameron, A. 1992. Controlling relative humidity in modified atmosphere packages of tomato fruit. HortScience 27: 336–339.

Soliday, C.L., Kolattukudy, P.E., and Davis, R.W. 1979. Chemical and ultrastructural evidence that waxes associated with the suberin polymer constitute the major diffusion barrier to water vapor potato tuber (*Solanum tuberosum* L.). Planta 166: 207–214.

Tatsumi, Y., Watada, A. and Wergin, W. 1991. Scanning electron microscopy of carrot stick surface to determine cause of white translucent appearance. J. Food Sci. 56: 1357–1359.

Tatsumi, Y.; Watada, A., and Ling, P. 1993. Sodium chloride treatment or waterjet slicing effects on white tissue development of carrot sticks. J. Food Sci. 58: 1390–1392.

Watada, A.E., Kazuhiro, A., and Yamuchi, N. 1990. Physiological activities of partially processed fruits and vegetables. Food Technol. 44: 116–122.

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Fresh Mushroom Quality as Affected by Modified Atmosphere Packaging

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ABSTRACT

The effect of O_2 concentration, at 2–6% CO_2, on shelf-life of fresh mushrooms in modified atmosphere packages (MAP) was studied. For conventional mushrooms optimum in-package O_2 was 6% to reduce cap development. Mushrooms irrigated with 0.3% calcium chloride during cropping had a higher rate of maturation and optimum O_2 was 2% during storage. At this low O_2 concentration, however, storage may be potentially hazardous and is not recommended. Surface moisture of mushrooms significantly increased during storage and may have obscured any beneficial effect of MAP on mushroom color. Precise control of relative humidity in the package is recommended.

Key Words: mushrooms, modified atmosphere, packaging, maturity index.

INTRODUCTION

SHELF-LIFE OF FRESH, commonly grown mushrooms (*Agaricus bisporus*) is usually limited to 1–3 days at ambient (ca. 22°C) temperature (Burton and Twyning, 1989). Controlled atmosphere (CA) storage is costly and not practical for short term storage of produce with short shelf-life, such as mushrooms. Any beneficial effects of CA storage are lost as soon as the produce is removed from CA. Modified atmosphere packaging (MAP) has continuing beneficial effects until the package is opened and can be very useful in extending the shelf-life of fresh produce. About 50% of the U.S. mushroom crop is produced in Pennsylvania and shipped throughout the country. (Agricultural Statistics Board, 1991). MAP can provide an economical and effective way of extending shelf life of fresh mushrooms during shipment and marketing.

There is little published consensus on the optimum CA to increase shelf-life of mushrooms. Gormley and MacCanna (1967) reported that the mushroom shelf-life could be increased by overwrapping with polyvinyl chloride (PVC) films. They attributed the benefits to conservation of water, but also indicate that the artificial atmosphere may cause chemical changes. Sveine et al. (1967) while investigating the storage life of mushrooms reported that high CO_2, low O_2, and low temperatures, prevented cap opening. In that work, N_2 with 0.1% O_2 and 5% CO_2 in storage was optimum for maximum shelf-life. Nichols and Hammond (1973) varied in-package gaseous concentrations in pre-packs stored at 2 and 18°C using different films. Packages with CO_2 of 10–12% and O_2 of 1–2% stored at 18°C resulted in mushrooms with slowest opening of the pileus and color deterioration. At 2°C, CO_2 and O_2 concentrations came to equilibrium at about 4–10% and 11–17% respectively, depending on film overwrap. At that temperature, mushrooms tended to discolor, which may have been due to the high CO_2. Murr and Morris (1974) reported that browning was slowed due to inhibition of enzyme (tyrosinase) by the high CO_2.

Murr and Morris (1975) reported that 0% O_2 retarded pileus expansion and stipe growth, while 5% O_2 promoted pileus expansion and stipe growth after 7 days at 10°C. CO_2 at 5% stimulated stipe elongation but suppressed cap growth. Burton et al. (1987) used a microporous with a relatively impermeable film to overwrap mushrooms. They reported a progressive reduction of mushroom development with lowering of O_2 from 14 to 4% and increase in CO_2 from 7 to 20% after 72 hr. Lopez-Briones et al. (1992) suggested that storage atmosphere should contain 2.5 to 5% CO_2 and 5 to 10% O_2. Beit-Halachmy and Mannheim (1992) reported that MAP seemed to have a beneficial effect on appearance and inferred that it may be due to a microstatic effect, since MAP did not affect rate of respiration.

In studies using MAP (Nichols and Hammond, 1973, 1974, 1976; Burton et al. 1987; Burton and Twyning, 1989; Beit-Halachmy and Mannheim, 1992), mushrooms have been packaged with either PVC film or its combination with a microporous film. Permeabilities for O_2/CO_2 of such films were near unity. As a result, at steady state, low O_2 concentration (<10%) was accompanied by high CO_2 (>10%) concentration.

Beelman et al. (1986, 1992) reported that addition of calcium chloride to irrigation water during cropping delayed cap opening and deterioration of external appearance. Solomon et al. (1991) reported that irrigation of mushrooms with 0.25% calcium chloride solution reduced browning but appeared to increase senescence. Bartley et al. (1991) studying mushroom production on a semi-commercial scale at the Mushroom Test and Demonstration Facility (MTDF) at Penn State reported a significantly improved color of mushrooms irrigated with 50 ppm Oxine plus 0.075% calcium chloride before harvest and during postharvest storage. Considering reported differences in postharvest qualities, our experiments included mushrooms watered with 0.3% calcium chloride solution. Our objective was to determine the effect of O_2, with a relatively low level of CO_2 concentration, on the shelf-life of mushrooms stored in MAP.

Table 1—Spawn type and flush at harvest of conventional and calcium chloride irrigated mushrooms used to study shelf-life in MAP

Replication	Irrigation treatment	Spawn type	Flush no.
1	Conventional	Alpha HOW #H2	1
2		Alpha HOW #H2	3
1	Calcium chloride	Hauser HOW #A93	2
2		Hauser HOW #A93	3

MATERIALS & METHODS

MUSHROOMS (*AGARICUS BISPORUS*) of U-1 (hybrid off-white) strain were grown on traditional horse manure-based compost in the MTDF at Pennsylvania State University. Spawn type and flush at harvest are shown in Table 1. Freshly harvested mushrooms, free of blotch, were transported within 1 hr after harvest and promptly placed in storage at 4°C before packaging.

Different amounts of mushrooms were used to create different steady state gaseous concentrations while using the same type and thickness of packaging film. Since the same size containers were used to package different amounts of mushrooms, each treatment had different void volume and time to attain the required atmosphere.

Packaging and storage

Mushrooms were sorted by size and appearance. Diseased, damaged, open-veiled and extremely large or small mushrooms (25 mm < cap

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diameter < 40 mm) were discarded. Stems were hand trimmed to stipe length 7 ± 2 mm. Different amounts of mushrooms (50 ± 5, 80 ± 5, 100 ± 5 and 120 ± 5 g) were weighed and placed into linear polystyrene mushroom trays (20 cm × 15 cm × 3.5 cm). The trays were inserted into 25cm × 17.5cm, 60 gauge (0.0015 cm) polyethylene pouches (D940, Cryovac Inc., Duncan, SC) and heat sealed to form a tight overwrap on the tray. Total volume of the packages remained constant throughout the study. Packages were stored at 12°C and 80% RH in a low temperature incubator (Model 815, Lunair Environmental, Inc., Williamsport, PA).

In-package O_2 and CO_2 concentrations

Gaseous concentrations in two packages/treatment were determined every day for 9 days using a gas chromatograph (GC) (Model 5890 Series II, Hewlett Packard Co., Avondale, PA) equipped with thermal conductivity detector. A drop of silicone glue was placed on the surface of a small patch of adhesive tape on pouch surface. The solidified drop provided a septum for gas sampling, ensuring reseal of the pouch after withdrawal of the sampling needle.

Gas samples (5 mL) were withdrawn using a syringe and injected into a 0.25 cc sample loop of the GC to ensure adequate flushing. A 3.2 mm stainless steel column packed with molecular Sieve 13X was used for O_2 and N_2 separation. Another stainless steel column, packed with Poropak Q was used for CO_2 separation. The column was maintained at 35°C and the detector at 200°C. Flow rate of the He carrier gas was 20 mL/min. The GC was connected to a Hewlett Packard (3396A) Integrator which recorded area of the peaks. Quantification of O_2 and CO_2 was by comparing peak areas of samples to standards. Experimentally determined in-package O_2 and CO_2 concentrations were compared with the predicted values obtained from the mathematical model described by Roy and Anantheswaran (1994).

Measurements of quality characteristics

Six mushrooms were removed at random to evaluate quality characteristics on day 0. Three trays of mushrooms from each weight category were removed at random from the low-temperature incubator after 3, 6 and 9 days and all mushrooms in each tray were evaluated. Mushrooms were weighed as a group immediately to estimate the fresh weight loss. The same mushrooms were then used to evaluate maturity, surface moisture and color.

Maturity index. The maturity index was assigned to mushrooms based on extent of cap opening on a 7 point scale as described by Guthrie (1984) (Table 2.)

Surface moisture. Surface moisture content of mushrooms was measured by a near-infrared technique described by Roy et al. (1993). Each mushroom was mounted on a sample holder after stipes were trimmed, and scanned 25 times using the NIR System 6500 monochromator (Perstorp Analytical, Silver Springs, MD). To obtain the spectrum baseline, a ceramic standard was scanned 8 times before and after each mushroom. Average reflectance of the mushroom was divided into the average reflectance of the ceramic reference and reported as log(1/Reflectance). The log(1/Reflectance) data were recorded at 2 nm intervals.

A calibration equation was developed using the spectra of mushrooms (stored in packages or open air) scanned at both 4°C and 12°C. Calibration was done both with and without scatter correction using the same wavelengths (600–2200 nm) and mathematical treatments as reported by Roy et al. (1993). (The standard error of prediction was 0.7 when the equation was validated using 10 mushrooms scanned at 12°C). The equation thus developed was used to fit the log (1/Reflectance) values of the spectra we obtained and thus predict surface moisture. Infrasoft International software (Infrasoft International, Port Matilda, PA) was used to collect and analyze data, perform calibration and cross-validation, and predict moisture content of the mushrooms (Shenk and Westerhaus, 1991).

Color. Surface color was measured using a Minolta Chroma Meter (CR-200; Dynamic Electronic Sales, Churchville, PA). Before measurement, the instrument was standardized with a standard white plate (Calibration Plate CR-A43). Three measurements were done at random locations on the cap of each mushroom and compared to the ideal mushroom (target) color values of L = 97, a = −2, and b = 0 (Ajlouni, 1991) using ΔE as described by the following equation:

$$\Delta E = [\{L - 97\}^2 + \{a - (-2)\}^2 + \{b\}^2]^{1/2} \quad (1)$$

ΔE indicates the degree of overall color change in comparison to color values of an ideal mushroom. ΔE and absolute L values were used to describe the color of mushrooms during storage.

Table 2—Classification of stages in sporophore development[a]

Stage	Description
1	Veil intact (tight)
2	Veil intact (stretched)
3	Veil partially broken (< half)
4	Veil partially broken (> half)
5	Veil completely broken
6	Cap open, gills well exposed
7	Cap open, gill surface flat

[a] Source: Guthrie (1984)

Statistical analysis

Studies with calcium-treated and conventional mushrooms were conducted as two separate experiments and therefore, no statistical comparison was made between these two treatments. Each experiment was analyzed as a four (treatments) by three (days) factorial model with replications as blocks. Data on color, maturity index, surface moisture content and weight loss by the mushrooms were subjected to analysis of variance (ANOVA). General Linear Model Procedure developed by Statistical Analysis System (SAS Institute, Inc., 1985) was used to perform ANOVA and to compare treatment means within each day. To compare rate of weight loss of mushrooms among different treatments, the slopes of weight loss vs time were compared. Bonferroni multiple comparison method ($p \leq 0.5$) was used to determine the difference between slopes and between treatments within each day (Neter et al., 1985).

RESULTS & DISCUSSION

In-package O_2 and CO_2 concentrations

The O_2 concentrations in packages containing 50, 80 and 100g mushrooms treated with or without calcium chloride were similar to predicted values (Roy and Anantheswaran, 1994) calculated during the first 5 days storage. After that measured O_2 concentrations were higher than predicted values (Fig. 1a, 1b). The rate of respiration of mushrooms in the mathematical model (for prediction) was determined within 24 hr of harvest. Any change in respiration rate during storage was expected to cause a deviation in measured gaseous concentration. Therefore, the higher in-package O_2 concentrations after 5 days storage were probably due to decreased respiration. Ajlouni (1991) also reported a decreased respiration rate of mushrooms after 4 days storage in air at 12°C.

Mushrooms stored in air had a respiratory rise after 3 days storage at 18°C (Hammond and Nichols, 1975) and at 12°C (Ajlouni, 1991). Similar O_2 concentrations between measured and predicted values for 50, 80 and 100g packages for the first five days implied that there was no respiratory rise in the mushrooms. Packages containing 120g mushrooms had lower O_2 concentrations than predicted at the end of 2 days storage, suggesting that respiration increased after 2 days.

A high ratio of CO_2 transmission rate (CTR) to O_2 transmission rate (OTR) for the film used prevented accumulation of high CO_2 levels in the package. The CO_2 concentration ranged from 2% in 50g packages to 6% in 120g packages (Fig. 2a, 2b). As expected, 120g packages had lower-than-predicted O_2 and higher-than-predicted CO_2 for both calcium chloride-treated and conventional mushrooms. Beit-Halachmy and Mannheim (1992) reported similar results and attributed the lower-than-predicted O_2 to microbial activity. This lower-than-predicted O_2 (and higher-than-predicted CO_2) in our results with 120g of mushrooms could be due to higher respiration rates. Microbial activity was not likely in 120g packages, since those containing 50, 80 and 100g mushrooms (with higher in-package O_2) would also have lower than predicted O_2. Microbial activity would be expected at higher O_2 and lower CO_2 concentrations (Brody, 1989).

Physical stresses, such as impact bruising, wounding, and tissue damage, stimulate respiration of fresh vegetables (Kader, 1987). Ajlouni (1991) found increased respiration rate of mushrooms when they had been subjected to stresses like irradiation and cutting of lower stipes. Researchers had indicated that mushrooms could tolerate CO_2 levels up to 50% (Sveine et al.,

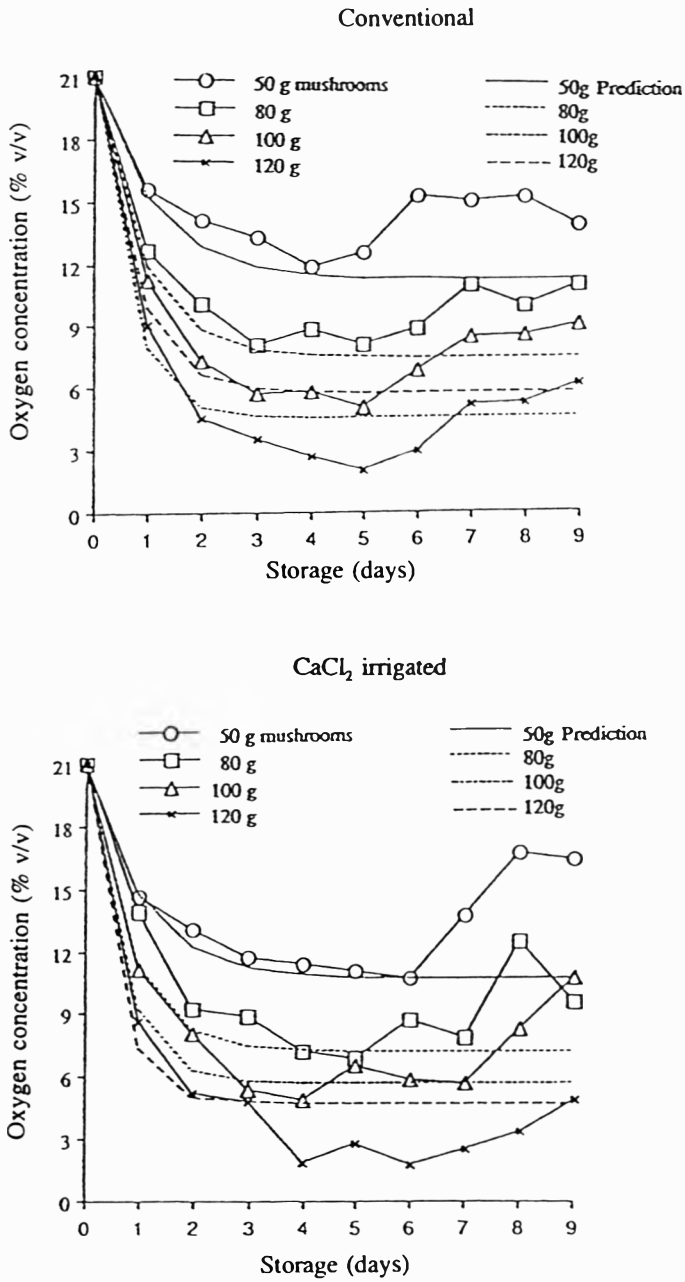


Fig. 1—In-package O_2 concentration attained with conventional or calcium chloride irrigated mushrooms at 12°C.

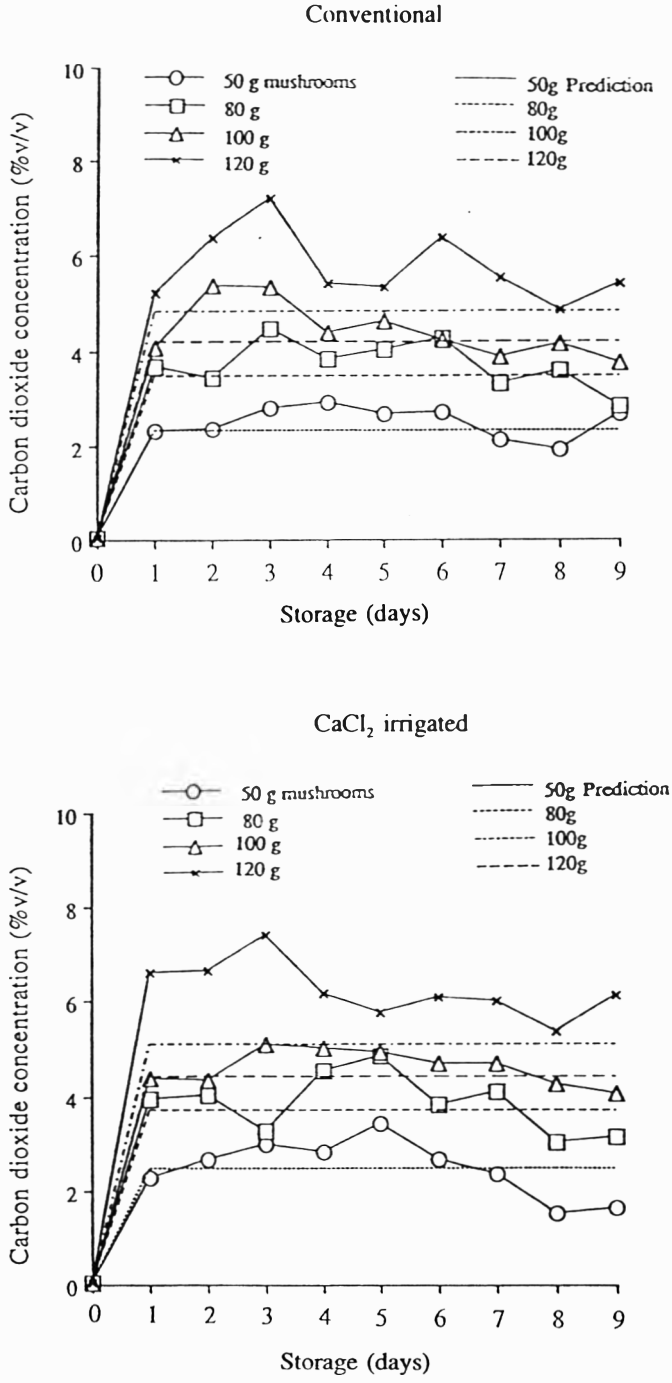


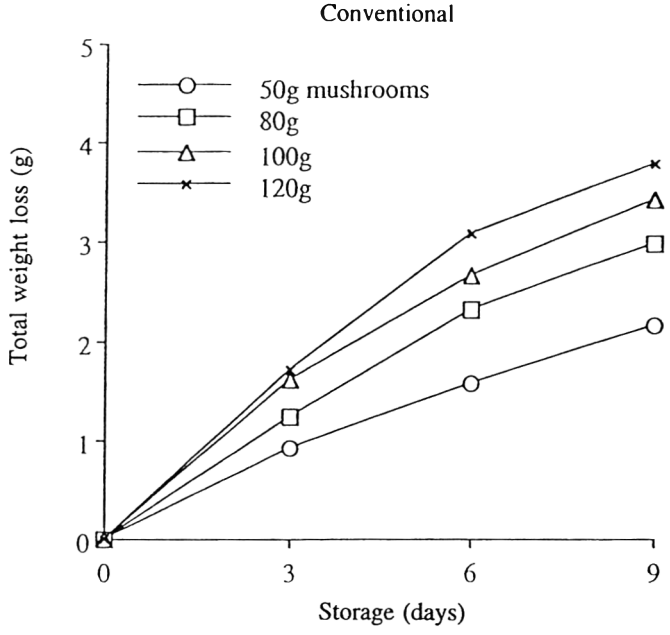
Fig. 2—In-package CO_2 concentration attained with conventional or calcium chloride irrigated mushrooms at 12°C.

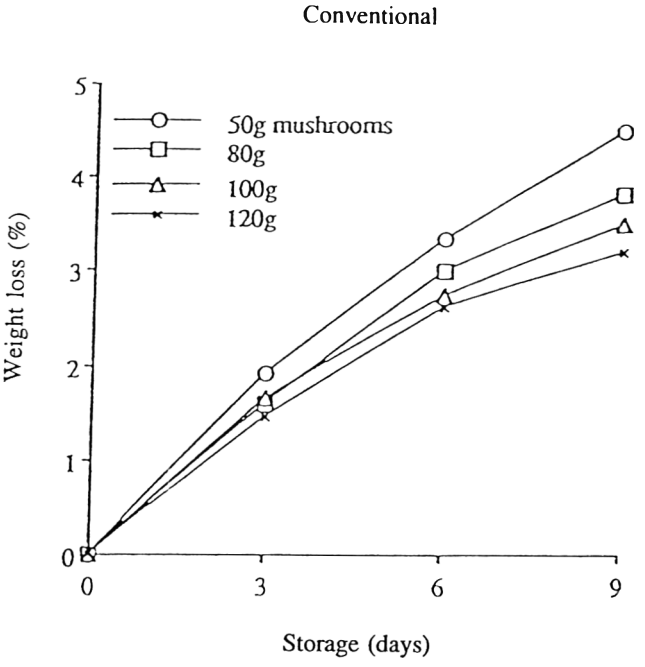
1967; Murr and Morris, 1974). However, according to Lopez-Briones et al. (1992), 5% or higher CO_2 would be phytotoxic. In our results, mushrooms in 120g packages attained an in-package CO_2 > 5% and therefore might have been under stress. Packages containing 50, 80 and 100g mushrooms probably had less than a phytotoxic level of CO_2 (Lopez-Briones et al., 1992). Another possibility for higher rates of respiration of mushrooms in 120g packages would be the Pasteur effect (Kays, 1991) due to anaerobic respiration.

Effect of MAP on shelf-life

Weight loss. Weight loss occurred in conventional mushrooms (Fig. 3a) and the % weight loss (Fig. 4a) varied from 3% in 120g packages to about 4.5% in 50g packages after 9 days storage. This was lower than reported weight loss of 5 to 7% after 5 days at 18°C (Nichols and Hammond, 1974). Higher weight loss in that study could be due to the higher temperature of storage, which increases water vapor transmission rate of films and transpiration and respiration rates of the mushrooms.

Mushrooms in 50g packages had less total weight loss than those in 100 and 120g packages throughout storage and lower total weight loss than those in 80g packages after 6 days ($p < 0.05$). The weight loss from the mushroom surface is affected by loss of water from the package to surrounding atmosphere, due to water vapor pressure difference across the packaging film, and to the loss of carbon upon formation of CO_2 during respiration. Mushrooms lack a protective epidermal structure to prevent excessive moisture loss and therefore have very high transpiration rates (San Antonio and Flegg, 1964). Low moisture permeation through the film (80g/m²/day; Cryovac, Duncan, SC) is, therefore, the rate-limiting step in weight loss. Since the rate of diffusion is dependent on vapor pressure differences, and not the amount of mushrooms in the package, the same rate of weight loss would be expected for different amounts of mushrooms, once the RH in the package became saturated. This as-





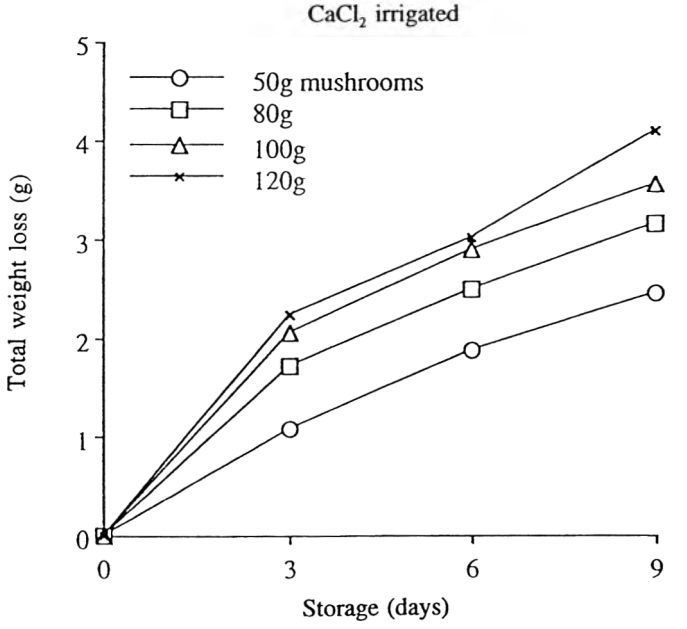


Fig. 3—Weight loss of mushrooms during storage in MAP at 12°C and irrigated with water (largest standard error of mean = 0.070), or 0.3% calcium chloride solution (largest standard error of mean = 0.057).

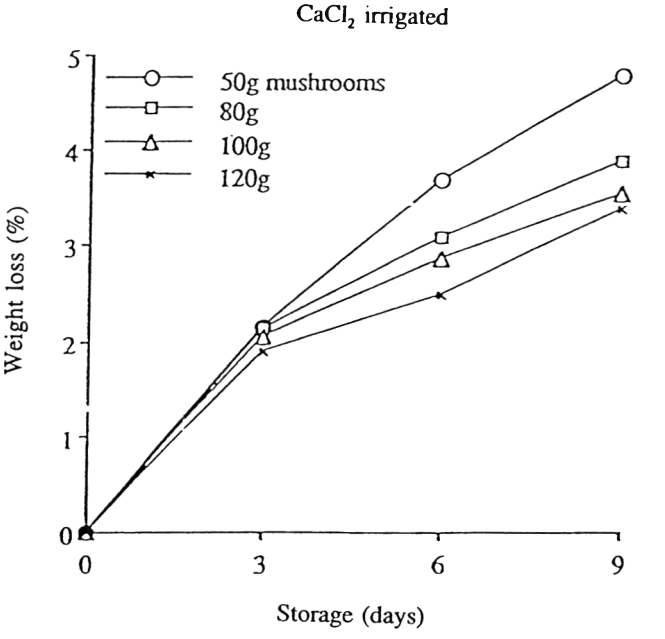


Fig. 4—Percent weight loss of mushrooms during storage in MAP at 12°C and irrigated with water (largest standard error of mean = 0.092), or 0.3% calcium chloride solution (largest standard error of mean = 0.095).

sumes that the weight loss due to loss in respiratory carbon is negligible. To determine whether there was a difference in rate of weight loss between packages, the slopes of curves (Fig. 3a) were compared using Bonferronni Multiple Comparison Test (Neter et al., 1985). The rate of weight loss of 50g mushrooms was lower than that of mushrooms in 80, 100 and 120g packages ($p < 0.05$). However, after 6 days storage, rates of weight loss were similar for mushrooms in packages of different amounts (Fig. 3a). These results indicate that packages containing 50g mushrooms were possibly unsaturated with water vapor during the first 6 days storage.

Similar weight loss was observed with calcium chloride-treated mushrooms during the first 6 days storage (Fig. 3b, 4b). Lower ($p < 0.05$) weight loss was also noticed for calcium chloride-treated mushrooms in 50g packages than in those with higher amounts of mushrooms throughout storage (Fig. 3b). Like conventional mushrooms (Fig. 3a), calcium chloride irrigated mushrooms in 50g packages had lower rates of weight loss than those in packages of higher amounts ($p < 0.05$).

Maturity. A decrease of maturity index was observed with decreased O_2 (and increased CO_2) in packages containing conventional mushrooms (Fig. 5a). Those in 50g packages (with minimum O_2 of 12%) had higher maturity index ($p < 0.05$) than those present in 100g (minimum O_2 of 6%) and 120g packages (with minimum O_2 of 2%) after 6 days storage. No significant differences in maturity index were observed between those in 80g packages (with minimum O_2 concentration of 8%) and those in 100 and 120g packages ($p > 0.05$). This implied that there was no further decrease in rate of senescence when mushrooms were stored in O_2 concentrations <5% in MAP at 12°C.

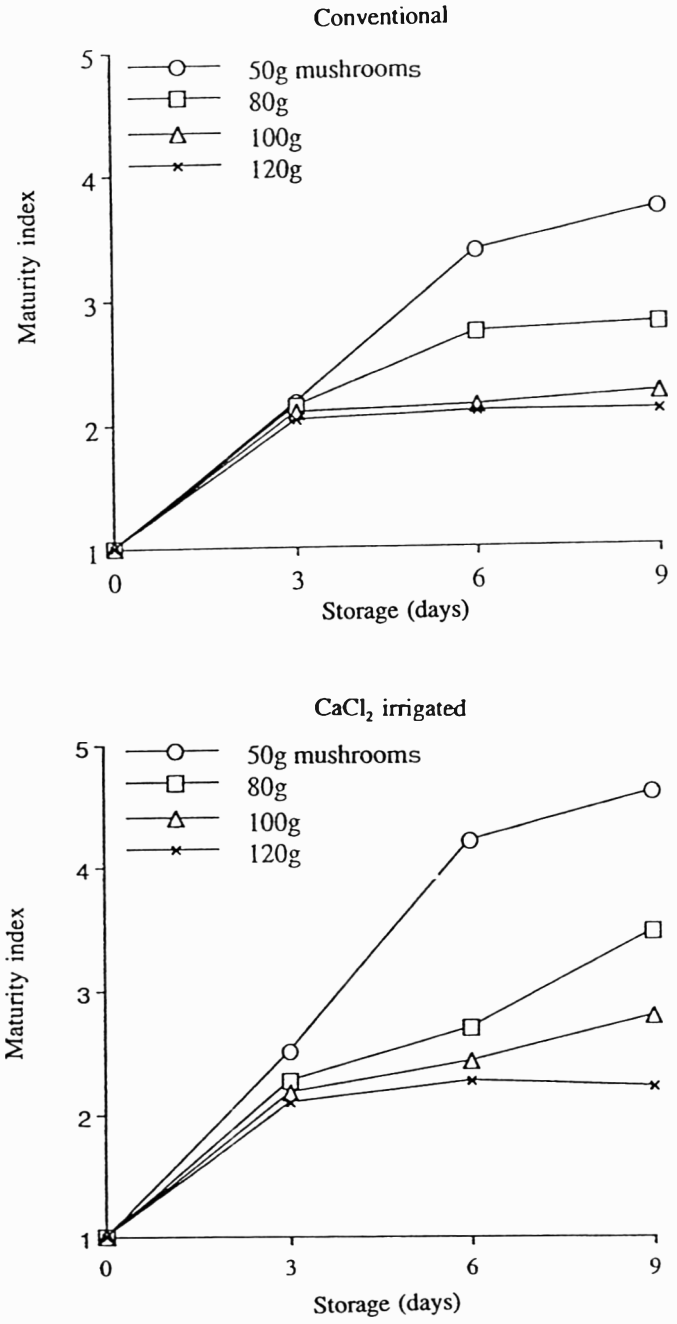


Fig. 5—Effect of MAP at 12°C on maturity index of mushrooms irrigated with water (largest standard error of mean = 0.0.142), or 0.3% calcium chloride solution (largest standard error of mean = 0.154).

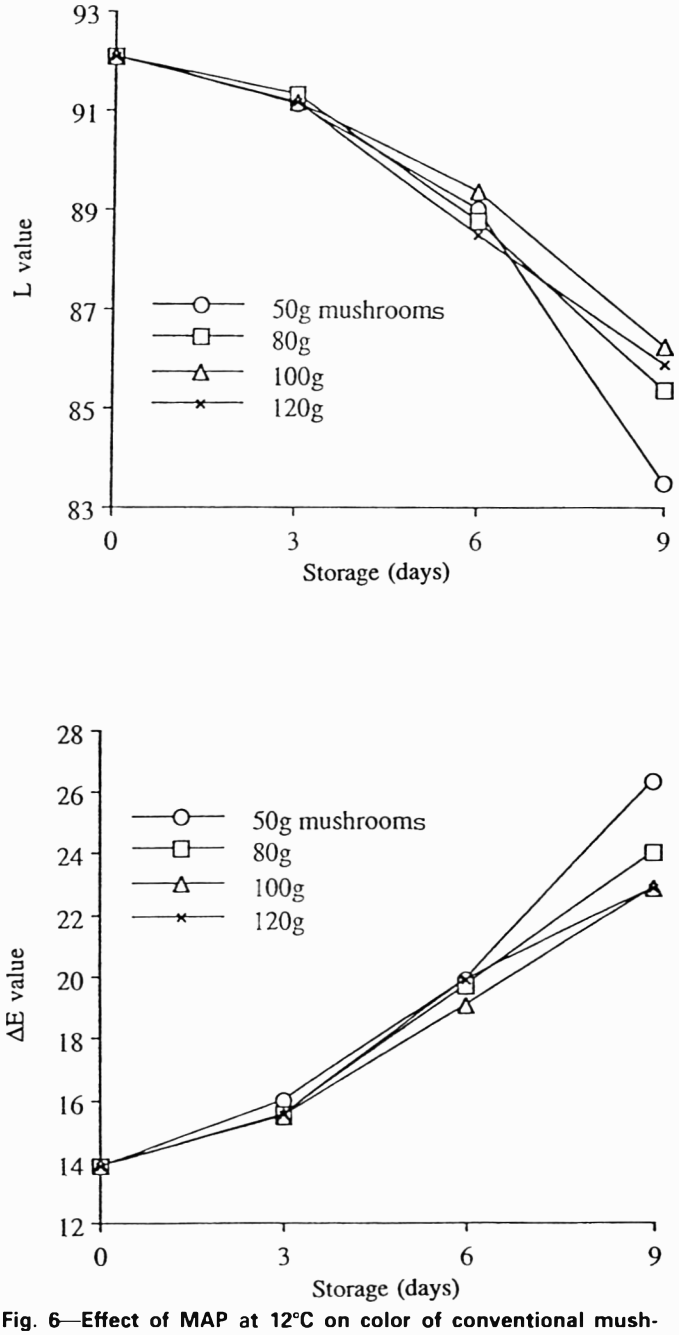


Fig. 6—Effect of MAP at 12°C on color of conventional mushrooms. L value (largest standard error of mean = 0.528), and ΔE value (largest standard error of mean = 0.560).

Murr and Morris (1975) stated that the cap opening of mushrooms in storage at 10°C was promoted by O_2 tensions lower than air and that the stimulatory effect was maximum when mushrooms were stored at 5% O_2. Several other researchers [Sveine et al. (1967) at 0–15°C; Nichols and Hammond (1973) at 18°C; Burton et al. (1987) at 18°C; Burton and Twyning, (1989) at 2–18°C], to the contrary, observed reduced rate of cap opening with decreased O_2 and increased CO_2. They attributed this effect to increased CO_2 rather than decreased O_2, since CO_2 is known to act as a regulator for mycelial growth and mushroom morphogenesis (Hammond and Wood, 1985). Our study at 12°C had a very low range of in-package CO_2 (2–6%). Burton et al. (1987) observed negligible differences in maturity index of mushrooms stored at 2% CO_2 from those stored at 6% CO_2 at 18°C. Burton and Twyning (1989) also reported no differences in cap development between mushrooms stored at 2% CO_2 and those stored at 3% CO_2 at 10°C. Therefore, we assumed that the effect of CO_2 was negligible and differences in maturity were probably due to differences in O_2 concentrations.

Lopez-Briones et al. (1992) reported retardation in cap development of mushrooms stored at 10°C in CA chamber at 15% CO_2, and the effect was not influenced by the O_2 in the chamber. However, they pointed out that $CO_2 \geq 5\%$ seemed to exhibit phytotoxicity as shown by increased respiration rate at the end of storage in high CO_2. Therefore, the effect of reduced cap development due to high CO_2 might be a physiological response to CO_2 stress rather than regulatory action of CO_2 in mushroom morphogenesis.

The harvested sporophore of the cultivated mushroom undergoes a course of development and senescence very similar to that of the growing fruit body (Hammond and Nichols, 1975). However, substrates of mycelial origin are no longer available for the cut sporophore which is therefore supported by substrates present at harvest (Bonner et al., 1956). Hence the respiration rate is important in determining onset of senescence in a cut

sporophore. Hammond and Nichols (1975) found that the rate of respiration of sporophores depended on the stage of cap development after harvest. In their study, respiration rate was high immediately after harvest, but fell rapidly during the first 5–10 hr of storage. Ajlouni (1991), however, did not report any initial decline in rate of respiration. Both researchers observed a rise in respiration within the first 3 days storage, which appeared to coincide with breaking of the velum and the phase of rapid gill development associated with cap opening. As described, the respiration rate of our mushrooms in 50, 80, and 100g packages did not increase during storage. The retardation of maturation at low O_2 concentration therefore, was due to lowered rates of respiration.

Mushrooms irrigated with 0.3% calcium chloride appeared to have higher rates of maturation compared to conventional mushrooms during storage in MAP (Fig. 5b). Similar to mushrooms irrigated with water, calcium chloride-treated mushrooms also showed decreased rate of senescence with decreased O_2 at 12°C. Mushrooms in 50 g packages (attaining a minimum in-package O_2 of 11%) showed higher maturity index than those in 120g packages (stored in minimum O_2 of 2%) after 6 days storage or those in 80g packages (with minimum O_2 of 7%) and 100g packages (with minimum O_2 of 5%) after 9 days storage ($p < 0.05$). Mushrooms in 80g packages had higher cap development than those in 120g packages after 9 days storage ($p < 0.05$).

Unlike conventional mushrooms, a very low O_2 concentration (<5%) was required to decrease the rate of cap development in mushrooms irrigated with calcium chloride. Barden (1987) and Solomon et al. (1991) also reported an increased rate of postharvest development of mushrooms irrigated with 0.25% and 0.5% calcium chloride. In higher plants, extracellular Ca^{2+} acts as an inhibitor of senescence by increasing membrane integrity and improving selective permeability of the cell (Ferguson, 1984).

Although minimum cap development was achieved with mushrooms in 120g packages, the center of these mushrooms was predicted to have an O_2 concentration < 0.25% (Roy and Anantheswaran, 1994). Sugiyama and Yang (1975) found toxin development by *Clostridium botulinum* in mushrooms stored in O_2 concentrations of 2%, as was found in 120g packages. Detectable toxin in their study was not produced unless a large number of spores were inoculated into the mushrooms. However, storing mushroom in such low O_2 concentrations could create a favorable micro-atmosphere at the center of mushrooms for growth and toxin production of the anaerobic spore former and therefore is not recommended.

Color. Many factors affect the color of mushrooms during storage. Reduced O_2 during storage can influence color by suppression of enzymatic browning and by reduction of microbial population (Beelman et al., 1989, Doores et al., 1987). No significant differences in color (L and ΔE) were observed between treatments for conventional (Fig. 6a, 6b) or calcium chloride irrigated (data not shown) mushrooms ($p > 0.05$). These result confirmed the findings of Murr and Morris (1974) that levels of $O_2 > 0\%$ had little or no effect in reducing discoloration and tyrosinase activity. Lopez-Briones et al. (1992) found no effect of CA in the range of 2.5% O_2 + 5% CO_2 or 15% O_2 + 20% CO_2 on the growth of fluorescent pseudomonads, including *Pseudomonas tolaasii*. Nichols and Hammond (1973) and Burton et al. (1987) reported an improvement in color of mushrooms stored in MAP. Burton et al. (1987) also reported an increase in disease symptoms by visual inspection of *Pseudomonas tolaasii* and *Aphanocladium album* growth with increased O_2 in the package. However, they attributed the effect to the suppressing action of high CO_2 rather than low O_2.

Surface moisture. An overall increase ($p < 0.05$) in the surface moisture occurred in conventional and calcium chloride-treated mushrooms after 9 days storage (Fig 7a & 7b) as measured by near infrared spectroscopy. The general consensus among mushroom growers and researchers (Gandy 1967; Sinden, 1971; Gaze, 1979; and Barber and Summerfield, 1990) is

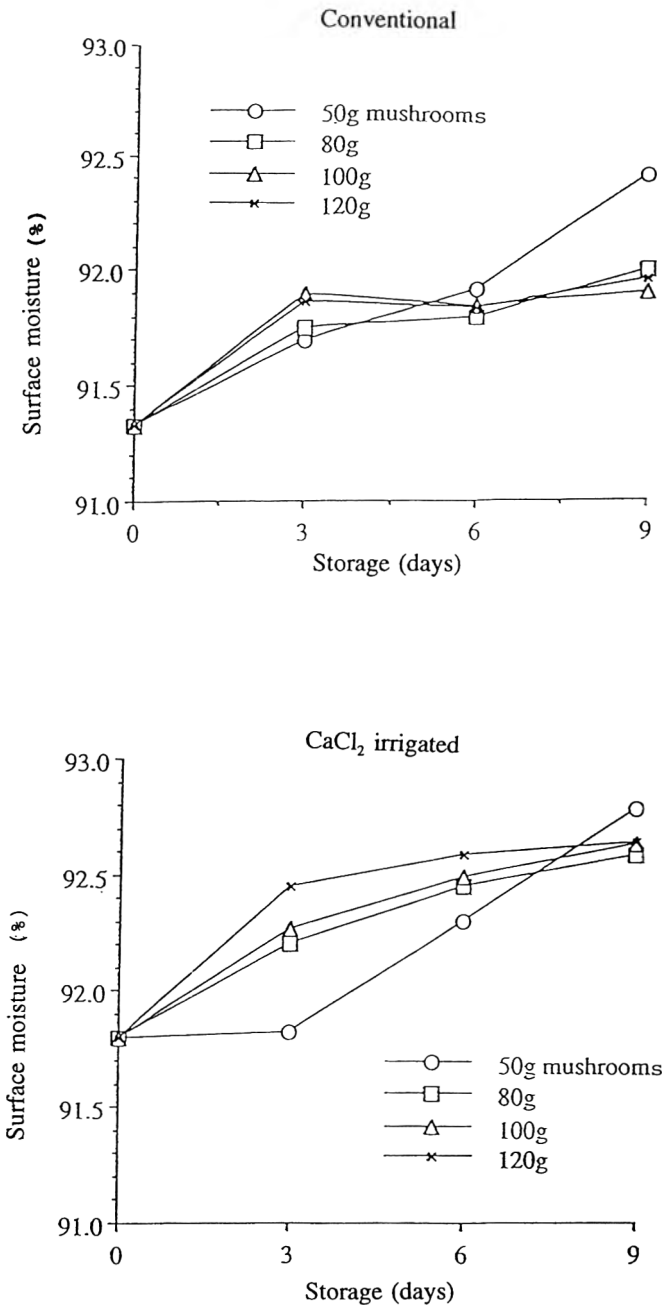


Fig. 7—Effect of MAP at 12°C on surface moisture (as determined by Vis-NIRS) of conventional mushrooms (largest standard error of mean = 0.053), and calcium chloride irrigated mushrooms (largest standard error of mean = 0.085).

that dry mushroom surfaces are the best prevention against bacterial blotch. Sinden (1980), and Barber and Summerfield (1990) reported that a wet surface reduces color perception because light is conducted deep into the cap and absorbed, whereas most light rays are reflected from a dry mushroom surface resulting in a higher L value. Therefore, lack of improvement in color of mushrooms in lowered O_2 might be due to decreased whiteness caused by increased surface moisture.

Conventional mushrooms stored in 50g packages had higher surface moisture after 9 days storage (Fig. 7a). The mushrooms in that package had a higher respiration rate (Roy and Anantheswaran, 1994), which resulted in more water being formed. Those mushrooms also lost more weight (percentage, Fig. 4a) due to higher moisture content, resulting in higher vapor loss through the package. However, no differences ($p > 0.1$) in surface moisture content of calcium chloride irrigated mushrooms at different O_2 concentrations were observed after 9 days storage (Fig. 7b).

CONCLUSIONS

DIFFERENT O_2 AND CO_2 COMPOSITIONS within the package can be brought about by using different amounts (50, 80, 100, and 120g) of mushrooms in MAP. Predicted O_2 and CO_2 concentrations were very similar to measured values in 50, 80, and 100g packages containing conventional or calcium chloride-treated mushrooms, until 5 days storage. Packages containing 120g of mushrooms (with and without calcium chloride treatment) had lower O_2 (and higher CO_2) than predicted. A progressive decrease in maturity index occurred with decreased O_2 in packages containing either conventional or calcium chloride irrigated mushrooms. Oxygen at 5% was optimum for retardation of cap opening of conventional mushrooms. Further reduction of O_2 did not reduce the maturity index. Oxygen at 2% was optimum for reduction of the maturity index of mushrooms when calcium chloride had been added to the irrigation water during cropping. This low O_2 concentration however, could promote growth and toxin production by *Clostridium botulinum* and therefore is not recommended. The range of O_2 concentrations did not affect color of mushrooms from either irrigation treatment.

REFERENCES

Agricultural Statistics Board. 1991. Mushrooms. National Agricultural Statistics Service, USDA, Washington, DC.

Ajlouni, S.O. 1991. Quality characteristics of two type hybrids of the cultivated mushrooms (*Agaricus bisporus*) and the improvement of their shelf life using stipe trimming and gamma irradiation. Ph.D. dissertation, Pennsylvania State University, University Park, PA.

Barber, W.H. and Summerfield, M.R.D. 1990. Environmental control of bacterial blotch on Pennsylvania shelf farms. Mushroom News 38(2): 8–17.

Barden, C.L. 1987. The effect of calcium chloride added to the irrigation water on the shelf life and quality of harvested mushrooms (*Agaricus bisporus*). M.S. thesis, The Pennsylvania State University, University Park, PA.

Bartley, C.E., Beelman, R.B., and Winnett, J.R. 1991. Factors affecting color of cultivated mushrooms (*Agaricus bisporus*) prior to harvest and during postharvest storage. Mushroom Sci. 13: 689–694.

Beelman, R.B., Quinn, J.J., Okereke, A., Schisler, L.C., Muthersbaugh, H.R., and Evensen, K. 1986. Effect of peat casing layer and the addition of calcium chloride to watering treatments on quality and shelf life of fresh mushrooms. In *Developments in Crop Science 10. Cultivating Edible Fungi*, P.J. Wuest, D.J. Royse, and R.B. Beelman (Ed.), p. 271–282. Elsevier Publ. Co., Amsterdam.

Beelman, R.B., Guthrie, B.D., and Royse, D.J. 1989. Influence of bacterial populations on the post-harvest deterioration of fresh mushrooms. Mushroom Sci. 11(2): 655–665.

Beelman, R.B., Bartley, C.L., Mau, J.L., Miklus, M.B., Solomon, J.M., Royse, D.J., Winner, J.R., and Muthersbaugh, H.R. 1992. Irrigation water treatments to improve quality and shelf life of *Agaricus* mushrooms. Mushroom News. 40: 18–23.

Beit-Halachmy, I. and Mannheim, C.H. 1992. Is modified atmosphere packaging beneficial for fresh mushrooms? Leb. Wes. U. Technol. 25(5): 426–432.

Bonner, J.T., Kane, K.K., and Levy, R.H. 1956. Studies on the mechanics of growth in the common mushroom (*Agaricus campestries*). Mycologia 48: 13–19.

Brody, A.L. 1989. *Controlled/Modified Atmosphere/Vacuum Packaging of Foods*, p. 179. Food & Nutri. Press, Inc., Trumbull, CT.

Burton, K.S., Frost, C.E., and Nichols, R. 1987. A combination of plastic permeable film system for controlling post-harvest mushroom quality. Biotechnology Letters 9(8): 529–534.

Burton, K.S. and Twyning, R.V. 1989. Extending mushroom storage-life by combining modified atmosphere packaging and cooling. Acta Horticulturae 258: 565–571.

Doores, S., Kramer, M., and Beelman, R.B. 1987. Evaluation of bacterial populations associated with fresh mushrooms (*Agaricus bisporus*). In *Developments in Crop Science, 10, Cultivating Edible Fungi* P.J. Wuest, D.J. Royse, and R.B. Beelman (Ed.), p. 283–294. Elsevier Publishing Co., Amsterdam.

Ferguson, I.B. 1984. Calcium in plant senescence and fruit ripening. Plant Cell & Environment 7: 477–489.

Gandy, D.C. 1967. The epidemiology of bacterial blotch of the cultivated mushrooms. Rep. Glasshouse Crops Res. Inst. 1966: 150–154.

Gaze, R.H. 1979. Report on ADAS farm investigation of bacterial blotch disease 1978/79. The Mushroom J. 83: 504–507.

Gormley, T.R. and MacCanna, C. 1967. Prepackaging and shelf life of mushrooms. Irish J. Agric. Res. 6(2): 255–265.

Guthrie, B.D. 1984. Studies on the control of bacterial deterioration of fresh, washed mushrooms (*Agaricus bisporus/brunescens*). M.S. thesis, The Pennsylvania State Univ., University Park, PA.

Hammond, J.B.W. and Nichols, R. 1975. Changes in respiration and soluble carbohydrates during the post-harvest storage of mushrooms (*Agaricus bisporus*). J. Sci. Food Agric. 26: 835–842.

Hammond, J.B.W. and Wood, D.A. 1985. In *The Biology and the Technology of the Cultivated Mushrooms*, P.B. Flegg, D.M. Spencer, & D.A. Wood (Ed.), p. 63–80. John Wiley & Sons, Ltd., UK.

Kader, A.A. 1987. Respiration and gas exchange of vegetables. In *Postharvest Physiology of Vegetables* J. Weichmann (Ed.), p. 597. Marcel Dekker, Inc., New York.

Kays, S.J. 1991. *Postharvest Physiology of Perishable Plant Products*, p. 532. Van Nostrand Reinhold, New York.

Lopez-Briones, G., Varoquax, P., Chambroy, Y., Bouquant, J., Bureau, G., and Pascat, B. 1992. Storage of common mushroom under controlled atmospheres. Int. J. Food Sci. Technol. 27: 493–505.

Murr, D.P. and Morris, L.L. 1974. Influence of O_2 and CO_2 on o-diphenol oxidase activity in mushrooms. J. Amer. Soc. Hort. Sci. 99(2): 155–158.

Murr, D.P. and Morris, L.L. 1975. Effect of storage atmosphere on post harvest growth of mushrooms. J. Amer. Soc. Hort. Sci. 100(3): 298–301.

Neter, J., Wasserman, W., and Kutner, M. 1985. *Applied Linear Statistical Models* (2nd ed.), p. 1127. Irwin, Homewood, IL.

Nichols, R. and Hammond, J.B.W. 1973. Storage of mushrooms in pre-packs: the effect of changes in carbon dioxide and oxygen on quality. J. Sci. Food Agric. 24: 1371–1381.

Nichols, R. and Hammond, J.B.W. 1974. Investigation on storage of prepacked mushrooms. Mushroom J. 24: 1–7.

Nichols, R. and Hammond, J.B.W. 1976. Storage of *Agaricus edulis* (syn. *A bitorquis*) sporophores in prepacks. Mushroom J. 45: 1–3.

Roy, S., Anantheswaran, R.C., Shenk, J.S., Westerhaus, M.O., and Beelman, R.B. 1993. Determination of moisture content of mushrooms by Vis-NIR spectroscopy. J. Sci. Food Agric. 63: 355–360.

Roy, S. and Anantheswaran, R.C. 1994. Mathematical modelling of oxygen and carbon dioxide concentrations in the package and within the fresh mushrooms stored in a modified atmosphere package. Int. J. Food Sci. Technol. (in review).

San Antonio, J.P. and Flegg, P.B. 1964. Transpiration from the sporophore of *Agaricus bisporus* 'white'. Amer. J. Bot. 51(10): 1129–1132.

SAS Institute Inc. 1985. *SAS User's Guide: Statistics, Version 5 Edition*. SAS Institute Inc., Cary, NC.

Shenk, J.S. and Westerhaus, M.O. 1991. Population definition, sample selection, and calibration procedures for near infrared reflectance spectroscopy. Crop Sci. 31: 469–474.

Sinden, J.W. 1971. Ecological control of pathogens and weedmolds in mushroom culture. Ann. Rev. Phytopathol. 9: 411–432.

Sinden, J.W. 1980. Strain adaptability. Mushroom News. 28: 18–32.

Solomon, J.M., Beelman, R.B., and Bartley, C.E. 1991. Addition of calcium chloride and stabilized chlorine dioxide to irrigation water to improve quality and shelf life of *Agaricus bisporus*. Mushroom Sci. 13: 695–701.

Sugiyama, H. and Yang, K.H. 1975. Growth potential of *Clostridium botulinum* in fresh mushrooms packaged in semipermeable plastic film. Applied Microbiol. 30(6): 964–969.

Sveine, E., Klougart, A. and Rasmussen, C.R. 1967. Ways of prolonging the shelf-life of fresh mushrooms. Mushroom Sci. 6: 463–474.

Antioxidant Activity and Total Phenolics in Different Genotypes of Potato (*Solanum tuberosum*, L.)

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ABSTRACT

Antioxidant activity of potato compared with that of broccoli, onion, carrot and bell peppers was higher than all except broccoli. Patatin appeared to be the major water-soluble compound that showed antioxidant activity. The activity varied among potato cultivars, but was not related to flesh color or total phenolics. Antioxidant activity was evenly distributed within tuber parts and/or sections, except for skin tissue which had the greatest antioxidant activity and total phenolic content. Total phenolics varied among cultivars, with some containing twofold higher concentrations than other cultivars. Phenolic content differences were genotype dependent and not related to flesh color.

Key Words: potato, antioxidants, phenolics, patatin

INTRODUCTION

ANTIOXIDANTS are important in prevention of pollution damage to plants, and disease prevention in both plants and animals. Potatoes differ in susceptibility to ozone, but endogenous antioxidant compounds can protect crops from injury and prevent yield reduction (Foster et al., 1983; Hofstra et al., 1983). Reactive oxygen molecules may cause damage when consumed by denaturing proteins, damaging nucleic acids and membranes (Byers and Perry, 1992). Oxidative and free radical processes have been related to cancer initiation and promotion (Kinsler, 1986). Damage can be prevented by dietary consumption of antioxidant compounds such as α-tocopherol (vitamin E), glutathione (GSH), β-carotene and ascorbic acid (vitamin C), Machlin and Bendich, 1987). Diets high in antioxidant vitamins may help reduce risk of such diseases (Block, 1992; Byers and Perry, 1992).

Antioxidants are found in many fruits and vegetables, and include GSH, ascorbic acid, α-tocopherol, β-carotene, chlorogenic acid, quercetin and other flavonoids (Jones et al., 1992; Larson, 1988). Potato is considered a good source of antioxidants such as ascorbic acid and α-tocopherol, which act synergistically (Byers and Perry, 1992). Potatoes also contain flavone aglycones, a major group of plant phenols which are potent antioxidants. Potato peel contains quercetin, a flavonol with antioxidant activity (Pratt and Watts, 1964). Such activity in flavonols is attributed to their action as free radical acceptors. Potato peel also contains chlorogenic acid, an effective antioxidant (Rodriguez de Sotillo et al., 1993). Potatoes (French fries and potato chips) are good sources of glutathione, reduced tripeptides, and reduced dithiothreitol (Jones et al., 1992). Glutathione is a water soluble antioxidant and anticarcinogen that helps maintain functional levels of other antioxidants such as vitamins C and E and β-carotene (Niki et al., 1982; Frei et al., 1988; Jones et al., 1992).

Potatoes are high in phenolic compounds which range from 530 μg/g to 1770 μg/g (Tripathi and Verma, 1975; Thomas and Joshi, 1977; Reeve et al., 1969). Phenols are important in protection and resistance of potato to soft rot caused by *Erwinia carotovora* bacteria and *Phytophthora* and *Phoma* exigua fungi (Kumar et al., 1992; Apomah and Friend, 1988). Natural resistance from phenolic compounds inhibits growth of pathogens in the field and storage, and has probably saved millions of dollars in crop loss (Kumar et al., 1991; Ampomah and Friend, 1988; Ghanekar et al., 1984). Also, the presence of phenolic compounds associated with glandular trichomes gives potatoes resistance to aphids (Lentini et al., 1990).

Antioxidants and phenolics are important in disease prevention in plants and animals, and information is limited on their composition and distribution in different potato cultivars. Our objectives were to (1) evaluate antioxidant activity and total phenolic distribution in potatoes, (2) determine if potato cultivars differ in antioxidant activity and total phenolic content, (3) identify compounds responsible for antioxidant activity, and (4) evaluate potato relative to selected vegetables for water-soluble antioxidant activity.

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MATERIALS & METHODS

Plant material

Two cultivars of yellow flesh (Granola and Yukon Gold) and white flesh (Viking and Russet Norkotah) potatoes were grown near Springlake, Texas in the summer of 1992. Three tubers were harvested from each cultivar, washed several times with distilled water and cut into apical (A), middle (M) and stem (S) parts. Each part was further partitioned into four sections, including (1) 10 mm of skin or periderm and cortex, (2) 9 mm of cortex and vascular ring, (3) 8 mm of medullary tissue, and (4) the next 7 mm of medullary tissue (Fig. 1). Fresh carrots (cv. Danvers 126), onions (cv. Texas Granex 1015), broccoli (cv. unknown), potatoes (cv. Yukon gold) and bell peppers (cv. unknown) were used for comparison of antioxidant activity.

Extraction

Potatoes and other vegetables were chopped into small pieces and homogenized in deionized water (0.25g tissue/mL water) for 30 sec using a Tissuemizer (Tekmar, Cincinnati, OH). Homogenate was filtered through miracloth (CalBiochem, San Diego, CA) into centrifuge tubes and centrifuged at 5,000 × *g* for 20 min. Supernatant was filtered through miracloth into test tubes. After capping, extracts in test tubes were boiled 5 min in a water bath to inactivate enzymes. After cooling in tap water, the solution was again filtered through miracloth into centrifuge tubes and centrifuged at 5,000 × *g* for 15 min. Supernatant was stored in capped tubes at −40°C. Supernatant aliquots were used for determination of antioxidant activity and total phenolic content.

Antioxidant procedure

Evaluation of antioxidant activity based on coupled oxidation of β-carotene and linoleic acid was conducted as described by Taga et al. (1984) with some modifications. β-carotene (2 mg) was dissolved in 20 mL of chloroform. A 3 mL aliquot of the solution was added to a conical flask with 40 mg linoleic acid and 400 mg Tween 40. Chloroform was removed with a rotary evaporator at 50°C. Oxygenated distilled water (100 mL) was added to the β-carotene emulsion and mixed well. Aliquots (3 mL) of the oxygenated β-carotene emulsion and 0.12 mL of the water soluble potato extract were placed in test tubes and mixed well. The tubes were immediately placed in a water bath and incubated at 50°C. Oxidation of the β-carotene emulsion was monitored spectrophotometrically by measuring absorbance at 470 nm. Sample absorbance was measured 10, 20, 30 and 40 min after addition of oxygenated water and incubation at 50°C. A control consisted of 0.12 mL distilled water, instead of potato extract. Degradation rate of vegetable extracts was calculated according to first order kinetics:

Table 1—Antioxidant activity of selected vegetables and potato components

Vegetable	Antioxidant activity[a]	Potato component	Antioxidant activity[a]
Bell Pepper	14.7d[2]	Chlorogenic acid (300 µg/mL)	25.2b
Broccoli	95.8a	Glutathione (100 µg/mL)	2.4c
Carrot	31.8c	Ascorbic acid (320 µg/mL)	−10.8d
Onion	24.7cd	Quercetin (15 µg/mL)	2.9c
Potato	68.7b	Patatin (3 mg/mL)	68.1a

[a] Antioxidant activity = % inhibition relative to control.
[b] Means within column with similar letters not significantly different (P< 0.05).

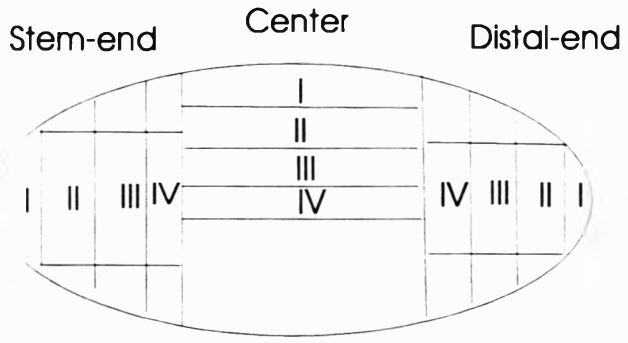

Fig. 1—Potato tuber parts and sections isolated for evaluation of total antioxidant activity and total phenolic content. Section I = 10 mm of skin and cortex. Section II = 9 mm cortex and vascular ring. Section III = 8 mm of medullary tissue. Section IV = 7 mm of medullary tissue.

$$\ln (a/b) \times 1/t = \text{sample degradation rate}$$

where: ln = natural log, a = initial absorbance (470 nm) at time 0; b = absorbance (470 nm) at 10, 20, and 30 min; t = time (min). Antioxidant activity (AA) was expressed as % inhibition relative to the control using:

$$AA = \frac{\text{Degradation rate of control} - \text{Degradation rate of sample}}{\text{Degradation rate of control}} \times 100$$

Total phenolics procedure

Total soluble phenolics in potato water extracts were determined using Folin-Ciocalteau reagent as described by Swain et al. (1959). Chlorogenic acid was the standard.

Antioxidant activity of potato components

Solutions of glutathione (100 µg/mL), ascorbic acid (320 µg/mL) and patatin (33 mg/mL) were prepared in distilled water. Solutions of chlorogenic acid (caffeolyquinic acid, 300 µg/mL) and quercetin (3,5,7,3'4'-pentahydroxyflavone, 15 µg/mL) were prepared in methanol. Aliquots (120 µL) were added to the oxygenated β-carotene emulsion and antioxidant activity was assessed.

Isolation of patatin

Patatin was purified from fresh tubers (cv. Superior) as described by Bohac (1991).

Statistical analysis

Values represent the mean of three replications, with each tuber or vegetable serving as a replication. Data were analyzed by analysis of variance ($P<0.05$), and mean separation was conducted using Duncan's multiple range test (SAS Institute, Inc., 1986).

RESULTS & DISCUSSION

POTATOES had exceptionally high antioxidant activity and were higher than all except broccoli (Table 1). They have been indicated as a source of water-soluble antioxidants (Pratt and Watts, 1964), but their high ranking among vegetable crops has not been emphasized. Potatoes are known to contain water-soluble antioxidants that act as free radical acceptors, e.g. glutathione, ascorbic acid, quercetin and chlorogenic acid (Pratt and Watts, 1964; Reeve et al., 1969; Munshi and Mondy, 1989; Jones et al., 1992). Antioxidant activity of vegetable extracts was expressed as % inhibition relative to a control which contained no plant extract. Minimal bleaching of the β-carotene emulsion occurred in samples treated with potato extract up to 40 min after addition of oxygenated water (Fig. 2).

Table 2—Antioxidant activity and total phenolic content of potato cultivars

	Flesh type	Antioxidant activity[a]	Total phenolics (µg/g)
Yukon Gold	Yellow	68.6b[b]	237.7d
Granola	Yellow	89.2a	407.0b
Russet Norkotah	White	88.1a	527.2a
Viking	White	65.2b	369.1c

[a] Antioxidant activity = % inhibition relative to control.
[b] Means within column with similar letters not significantly different (P< 0.05).

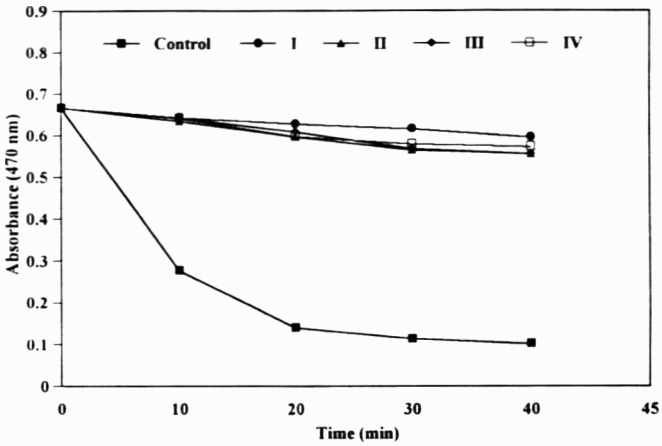

Fig. 2—Typical change in color of β-carotene/linoleic acid emulsion and different potato extracts. Section I = 10 mm of skin and cortex. Section II = 9 mm cortex and vascular ring. Section III = 8 mm of medullary tissue. Section IV = 7 mm of medullary tissue.

Potato cultivars differed in antioxidant activity (Table 2). Granola (yellow flesh) and Russet Norkohtah (white flesh) had greater antioxidant activity than Viking (white flesh) and Yukon Gold (yellow flesh), which indicated that carotenoid pigments were probably not responsible for much of the antioxidant activity. Carotenoids are effective antioxidants, but are not water soluble (Byers and Perry, 1992). Antioxidant distribution within the tuber was similar in the center and distal end, but was lower in the stem end than in the center (Table 3). Antioxidant activity within potato parts (distal, center and stem) was similar except for section I (skin and cortex) which had greater activity than sections II and III (cortex and medullary tissue). Antioxidant activity in the distal end of Yukon Gold was higher than the center and stem end (Table 4). However, in Viking and Granola, the center was higher than the stem end. In Russet Norkotah, no difference in antioxidant activity was observed among tuber parts. Minor differences in antioxidant activity were observed between sections within each genotype. In Yukon Gold, section I (skin and cortex) had higher activity than sections II, III and IV. No differences in antioxidant activity among sections were found in other genotypes.

Major differences in phenolic content were observed among potato cultivars (Table 2). Russet Norkotah had the highest total phenolic content, followed by Granola, Viking and Yukon Gold. Total phenolics in distal, center and stem ends exhibited wide variation within tubers (Table 3). A decreasing gradient in phenolic concentration was observed from stem to distal end which corroborated findings of Reeve et al. (1969). Minor differences in phenolic concentration were observed among sections, except for section I (skin and cortex) which contained greater levels

Table 3—Antioxidant activity and total phenolic distribution in potato tubers

Part	Antioxidant activity[a]	Total phenolics (μg/g)	Section[b]	Antioxidant activity	Total phenolics (μg/g)
Distal-end (A)	79.0ab[c]	353.7c	I	83.2a	430.2a
Center (M)	80.5a	385.0b	II	75.8b	364.9b
Stem-end (S)	73.4b	417.2a	III	74.4b	373.7b
			IV	76.7ab	372.2b

[a] Antioxidant activity = % inhibition relative to a control.

[b] Section I = 10 mm of skin or periderm and cortex. Section II = 9 mm cortex and vascular ring. Section III = 8 mm of medullary tissue. Section IV = 7 mm of medullary tissue.

[c] Means within column with similar letter not significantly different (P<0.05).

Table 4—Antioxidant activity and total phenolic distribution in distal, center, and stem ends of potato tubers as related to genotype

	Tuber parts					
	Distal-end	Center	Stem-end	Distal-end	Center	Stem-end
	Antioxidant activity[a]			Total phenolics (μg/g)		
Yukon gold	74.0a[b]	68.8a	63.0a	206.5b	239.9ab	266.7a
Granola	93.3a	92.3a	81.9b	361.2b	399.8b	460.1a
Russet Norkotah	84.3a	86.0a	93.4a	505.4a	526.3a	553.1a
Viking	64.2ab	76.4a	55.1b	341.5a	376.9a	388.8a

[a] Antioxidant activity = % inhibition relative to a control.

[b] Means within row with similar letters not significantly different (P<0.05).

Table 5—Antioxidant activity and total phenolic distribution in potato tuber sections as affected by genotype

	Sections[a]							
	I	II	III	IV	I	II	III	IV
	Antioxidant activity[b]				Total phenolics (μg/g)			
Yukon gold	81.3a[c]	65.0b	60.7b	67.4ab	271.0a	229.8ab	224.7b	225.4b
Granola	90.4a	89.0a	88.1a	89.2a	483.4a	373.7b	387.1b	383.9b
Russet Norkotah	90.4a	86.4a	86.7a	89.2a	560.6a	509.6a	520.6a	520.2a
Viking	70.8a	63.0a	62.0a	65.1a	405.9a	346.6a	362.4a	361.4a

[a] Section I = 10 mm of skin or periderm and cortex. Section II = 9 mm cortex and vascular ring. Section III = 8 mm of medullary tissue. Section IV = 7 mm of medullary tissue.

[b] Antioxidant activity = % inhibition relative to a control.

[c] Means within row with similar letters not significantly different (P<0.05).

than other sections (Table 3). The stem end of Yukon Gold and Granola had higher phenolic content than the distal ends (Table 4). Total phenolic content within sections of each cultivar were compared (Table 5). In Yukon Gold and Granola, section I (skin and cortex) had higher total phenolic content than other sections, while sections II, III and IV contained similar levels of total phenolics. Levels of total phenolics we found were lower than those previously reported (Tripathi and Verma, 1975; Thomas and Joshi, 1977; Reeve et al., 1969). Differences may be attributed to genetic and environmental conditions in which the potatoes were grown. Correlation analysis between antioxidant activity and total phenolic content showed a low positive correlation ($R^2 = 0.31$, $p = 0.0001$), indicating phenolics were a minor contributor to antioxidant activity in potato cultivars. Large variability in antioxidant activity indicated that the trait is related to genetics, implying a potential to breed potatoes with higher antioxidant activity.

Antioxidant activity of water-soluble compounds present in potatoes were compared (Table 1). Concentrations reflect amounts typically found in fresh potatoes. Patatin at 33 mg/mL (Storey and Davies, 1992) exhibited antioxidant activity that was very similar to that in potato extracts. Patatin is a water-soluble glycoprotein that can account for up to 40% of the total soluble protein in potato (Park, 1983). Soluble protein is evenly distributed in potato tubers (Neuberger and Sanger, 1942; Burton, 1966), which may account for minor differences within tubers. Cortex tissue is reported to have higher soluble protein content than pith (Munshi and Mondy, 1989), which is consistent with section I (skin and cortex) having higher antioxidant activity than section III (8 mm medullary tissue). Other plant proteins are reported to have antioxidant activity (Pratt, 1972). Chlorogenic acid at 300 μg/mL (Reeve et al., 1969) also had significant antioxidant activity. Chlorogenic acid extracted from potato peel was an effective inhibitor of lipid oxidation (Rodriguez de Sotillo et al., 1993). Chlorogenic acid is especially abundant in potato peel, which was consistent with section I (skin and cortex) having greater antioxidant activity and total phenolic content than other sections. Glutathione at 100 μg/mL (Jones et al., 1992) and quercetin at 15 μg/mL (Pratt and Watts, 1964) exhibited minor antioxidant activity. These compounds have been reported to have antioxidant and anticarcinogenic properties (Jones et al., 1992; Leighton et al., 1992). Ascorbic acid at 320μg/mL (Storey and Davies, 1992) promoted bleaching of the β-carotene emulsion. Ascorbic acid is a potent reducing agent and acts as a free radical scavenger (Niki, 1991). However, in the presence of metals, it may act as a prooxidant (Bendich et al., 1986). Metal contamination of water used in the antioxidant assay may have contributed to the prooxidant effect.

REFERENCES

Ampomah, Y.A. and Friend, J. 1988. Insoluble phenolic compounds and resistance of potato disc to Phytophthora and Phoma. Phytochemistry 27: 2533–2541.

Bendich, A., Machlin, L.J., Scandurra, O., Burton, G.W., and Wayner, D.D.M. 1986. The antioxidant role of vitamin C. Adv. Free Rad. Biol. Med. 2: 419–444.

Block, G. 1992. The data support a role for antioxidants in reducing cancer risk. Nutr. Rev. 50: 207–213.

Bohac, J.R. 1991. A modified method to purify patatin from potato tubers. J. Agric. Food Chem. 39: 1411–1415.

Burton, W.G. 1966. The potato: A survey of its history and the factors influencing its yield, nutritive value, quality and storage. In *European Association of Potato Research*, H. Veenman and N.V. Zonen (Ed.), Wageningen.

Byers, T. and Perry, G. 1992. Dietary carotenes, vitamin C, and vitamin E as protective antioxidants in human cancers. Annu. Rev. Nutr. 12: 139–159.

Foster, K.W., Guerard, J.P., Oshhima, R.J., Bishop, J.C., and Timm, H. 1983. Differential ozone susceptibility of Centennial Russet and White Rose potato as demonstrated by fumigation and antioxidant treatment. Am. Potato J. 60: 127–139.

Frei, B., Stocker, R., and Ames, B.N. 1988. Antioxidant defenses and lipid peroxidation in human blood plasma. Proc. Natl. Acad. Sci. USA. 85: 9748–9752.

Ghanekar, A.S., Padwal-Desal, S.R., and Nadkarni, G.B. 1984. The involvement of phenolics and phytoalexins in resistance of potato to soft rot. Potato Res. 27: 189–199.

Hofstra, G., Wukasch, R.T., and Drexler, D.M. 1983. Ozone injury on potato foliage as influenced by the antioxidant EDU and sulphur dioxide. Can. J. Plant Path. 5: 115–119.

Jones, D.P., Coates, R.J., Flagg, E.W., Eley, J.W., Block, G., Greenberg, R.S., Gunter, E.W., and Jackson, B. 1992. Glutathione in foods listed in the National Cancer Institute's health habits and history food frequency questionnaire. Nutr. Cancer 17: 57–75.

Kinsler, T.W. and Taffe, B.G. 1986. Free radicals in tumor promotion. Adv. Free Rad. Biol. Med. 2: 347–388.

Kumar, A., Pundhir, V.S., and Gupta, K.C. 1991. The role of phenols in potato tuber resistance against soft rot by *Erwinia carotovora* ssp. carotora. Potato Res. 34: 9–16.

Larson, R.A. 1988. Review article number 30: The antioxidants of higher plants. Phytochemistry 27: 969–978.

Leighton, T., Ginther, C., Fluss, L., and Harter, W.K. 1992. Molecular characterization of quercetin and quercetin glycosides in Allium vegetables: Their effects on malignant cell transformation. In *Phenolic Compounds in Food and Their Effects on Health II: Antioxidants and Cancer Prevention*, M.T. Huang, C.T. Ho, and C.Y. Lee (Ed.), p. 220–238. ACS Symposium Series 507, American Chemical Society, Washington, DC.

Lentini, Z., Earle, E.D., and Plaisted, R.L. 1990. Insect-resistant plants with improved horticultural traits from interspecific potato hybrids grown *in vitro*. Theor. Appl. Genet. 80: 95–104.

Machlin, L.J. and Bendich, A. 1987. Free radical damage: Protective role of antioxidant nutrients. FASEB J. 1: 441–445.

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Microwave Pre-treatment for Sun-Dried Raisins

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ABSTRACT

Commercial sun-drying of raisins requires a long time, and chemical pretreatments are used to increase the drying rate. The feasibility of improving the sun-drying process of grapes by microwaves was investigated. Sultana seedless grapes, dipped in alkali solution, were pretreated in a domestic microwave oven and dried by direct solar radiation. Microwave pre treatment reduced the moisture content by 10–20%. The microwave-treated grapes dried nearly two times faster than the controls. Blanching in boiling water had the same effect on the drying rate as microwaves. Color and appearance of treated grapes were comparable to commercial products.

Key Words: raisins, grapes, sun drying, microwave pre-treatment

INTRODUCTION

RAISINS ARE PRODUCED by sun-drying special cultivars of grapes, like Sultana seedless (light-colored) and Corinth currants (black-colored). The grape bunches, spread on cloth or in paper boxes, or hung under a transparent plastic film, are exposed to direct sunlight. Drying proceeds slowly for 2–3 wks until a moisture content of about 16% is reached (Somogyi and Luh, 1986). The long drying time is undesirable for economic reasons and because of the danger of contamination and spoilage of the product exposed to the open environment. The slow drying rate of grapes is caused by low water diffusivity in the flesh and low water permeability of grape skin (Raouzeos and Saravacos, 1986). The drying rate can be increased by methods removing the surface resistance of the grapes, e.g. by chemical pre treatments, such as dipping in solutions of alkali or ethyl oleate (Saravacos et al., 1988). The transport of moisture in food systems is usually controlled by diffusion of water molecules within the mass of material (Saravacos, 1986). Moisture diffusivity depends strongly on the physical structure of the material. Low diffusivities are found in gelatinized and sugar-containing foods, while moisture diffuses much faster in porous materials. Treatments of foods that increase porosity (void fraction), such as puffing, can increase substantially the drying rate of products (Marousis et al., 1991).

Microwave heating has been applied to some special drying operations, where the normal heat transfer mechanisms of convection and conduction are rate-limiting. Microwave or dielectric heating of food materials is based on the higher dielectric constant of water, compared to other components (Owusu-Ansah, 1981). The high absorption of microwave energy by water molecules results in higher heating and drying rates of the food (Schiffmann, 1987; Rosenberg and Bogl, 1987). Microwave or dielectric energy has found limited applications in food processing due to the relatively high cost of equipment and technical problems of processing operations (Anonymous, 1989; Giese, 1992; Schiffmann, 1992). By contrast, microwave ovens have been used extensively for quick heating of foods at home.

Due to the high cost, microwave energy can not compete with conventional air-drying. However, microwaves may be advantageous in the last stages of air-drying, when conventional drying is very slow. Microwaves can improve the rehydration capacity of some dried foods, by increasing their bulk porosity (puffing). Huxsoll and Morgan (1968) reported a combination drying process in which diced potato and apples were air-dried to about 50% moisture content and then microwave-dried in air or vacuum, resulting in puffed products of improved rehydration quality.

There is a definite need for increasing drying rate and improving the quality of sun-dried raisins. Little has been reported on the combination of microwaves with sun-drying of food products. Our objective study was to explore the feasibility of improving the sun-drying process by microwave pre treatment of grapes.

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Table 1—Pretreatment of Grape Samples

Sample no.	Alkali dipping (min)	Blanching time (min)	Microwave power (watts)	Microwave time (min)
S1	1	—	M (325)	0.5
S14	1	—	M (325)	1
S41	1	—	L (215)	2
S42,S16	1	—	M (325)	2
S43	1	—	H (430)	1
S44	—	0.5	—	—
S45	—	0.5	L (215)	2
S46	1	0.5	L (215)	2
S47	1	—	—	—
S48,S110	—	—	—	—

MATERIALS & METHODS

Grapes

Sultana seedless grapes, produced in the Corinth district of Greece, were harvested at maturity normal for raisin processing. The berries were separated by hand from the stems and they were kept refrigerated at 5°C before pre treatment and sun-drying. The berries with their cap-stems left on, had an ellipsoid shape with length 15-18 mm and diameter 12-14 mm. The average berry weight was 1.28 g. All berries of a sample were taken from the same grape cluster, which weighed 800–900 g.

Dipping and blanching

Each experimental sample of grapes consisted of about 100 g of berries of about the same size. Except for the control and two of the three blanched samples, all other grapes were prepared by dipping in a solution of 2.5% K_2CO_3 + 0.5% olive oil for 1 min. Alkali dipping is used commercially to 'check' the skin of the grapes and increase drying rate (Raouzeos and Saravacos, 1986). Thermal blanching of the grapes was carried out by immersing the berries in boiling water for 0.5 min, followed by cooling in chilled water to room temperature (≈23°C).

Microwave pre-treatment

Fresh, dipped or blanched grapes were treated in a microwave oven before sun-drying. A domestic microwave oven, Sharp model R-6280 (B), maximum nominal power output of 650 W at 2450 MHz, was used. A single layer of about 100 g of grape berries was placed in a ceramic plate and microwave-treated for 0.5 to 2 min at of the following nominal power settings: 215 W, 325 W or 420 W, indicated as treatments, l (low), m (med), and h (high) respectively. The power output was regulated by on-off control. The grape samples were weighed before and after microwave treatment to estimate moisture loss. Samples were rotated during treatment for even absorption of microwave energy (Table 1).

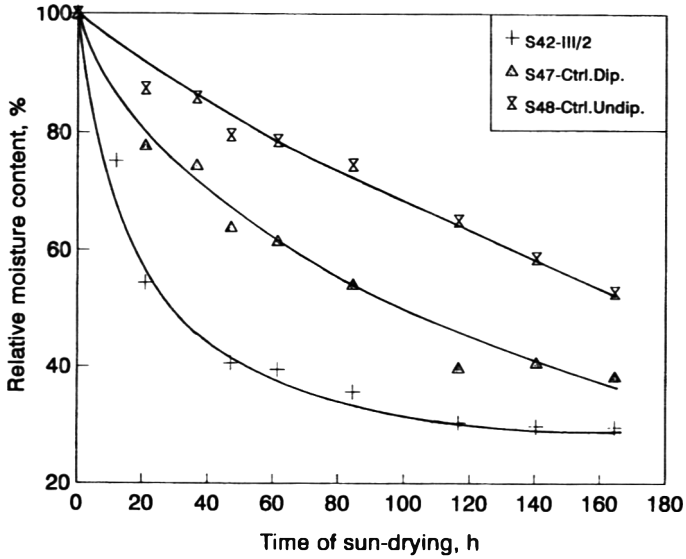

Fig. 1—Effect of alkali dipping and microwave treatment on the sun-drying rates of Sultana grapes. S42 Microwave treatment: 325 W (M), 2 min.

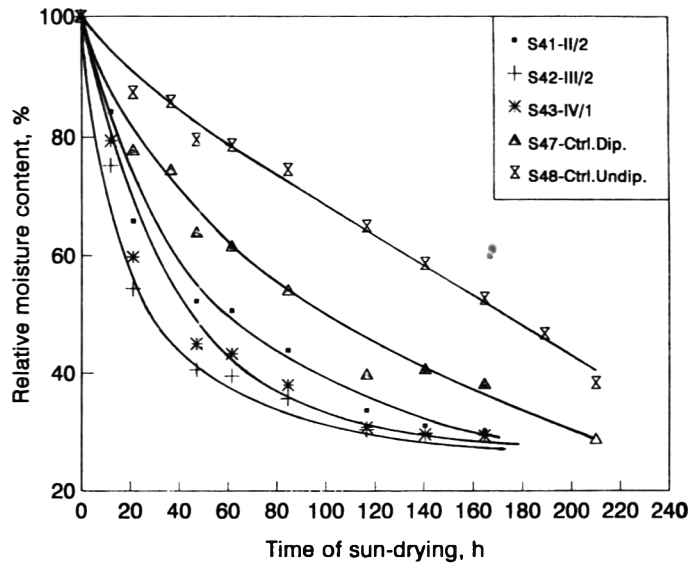

Fig. 2—Sun-drying curves of microwave-treated Sultana grapes: S41: 215W (L), 2 min. S42: 325 W (M), 2 min. S43: 430 W (H), 1 min.

Table 2—Reduction of moisture content of grapes due to microwave treatment

Microwave treatment (min)	Water loss %		
	Microwave power setting		
	L (215W)	M (325W)	H (420W)
0.5	—	6.6	14.7
1.0	—	18.8	14.7
2.0	15.6	35.7	—
3.5	33.3	—	—

Sun-drying

Treated and control grapes were placed in ceramic plates and sun-dried by direct exposure to solar radiation. Average daylight solar radiation at the site of the experiments during September was 500 W/m^2 and average daylight temperature was 22°C. The loss of moisture during drying was recorded by weighing samples periodically in a Mettler balance. Drying was completed after raisins reached the normal moisture content of commercial products, i.e. about 16% wet basis. The moisture content was determined by drying samples in a vacuum oven at 70°C for 24 hr, and it was used to calculate moisture content on dry basis (kg water/kg dry matter).

Color and water activity of raisins

The color of sun-dried raisins was determined with a Minolta Colorimeter, model CR-100. The raisins were placed in cups of 5 cm diameter and 3 cm depth. Every sample was pressed sufficiently, so that the surface appeared flat. Three measurements were made at different points of the sample. Cups were reloaded and 3 additional measurements were made. The procedure was repeated 7 times, so that 21 measurements were made on each sample. The appearance (shape and characteristic structure) of the raisins was determined by visual inspection and color photographs of single layers of dried products.

The sorption isotherms of the raisin samples at 25°C were determined by the gravimetric method of COST 90 (Wolf et al., 1984).

RESULTS & DISCUSSION

RESULTS from our preliminary investigation demonstrate the importance of microwave treatment on drying rate and quality of sun-dried raisins. Dipping Sultana grapes in an alkaline solution of 2.5% K_2CO_3 + 0.5% olive oil increased the drying rate significantly. The alkali dips of various combinations were used commercially to improve sun-drying of raisins (Raouzeos and Saravacos, 1986).

In our experiments, effective drying time was reduced by 45% with alkali dipping. Effective drying time is defined here as the time required to dry grapes from the initial moisture content of the sample (e.g. 75% wet basis or 3.0 kg water/kg dry matter, dry basis) to the normal moisture content of commercial products (16% wet basis or 0.2 dry basis). Sun-drying curves of dipped and control (undipped) grapes (Fig. 1) showed the effective drying time was reduced from 290 to 160 hr by alkali dipping. The increase of drying rate by alkali/oil treatment may be caused by loosening the skin (cuticle) structure of waxy materials and skin polymers (Saravacos et al., 1988).

Microwave pre treatment of alkali-dipped grapes for short times (1 or 2 min) resulted in a loss of 10–20% moisture. In the range of microwave power-exposure time we used, the loss of moisture (dry basis) increased almost linearly with the power output (Table 2). The sun-drying time of the treated grapes was reduced in all experimental runs. Thus, pre treatment l (215 W) for 2 min reduced the effective drying time from 160 to 140 hr. By raising the microwave power to 325 W (treatment m), effective drying time was reduced to 105 hr, i.e. a 40% reduction from the alkali dipped control (Fig. 1, 2).

The reduction of sun-drying time by microwave pre treatment may be caused by removal of 10–20% moisture during pre treatment. Also a change of the physical structure may occur in the grape berries. The absorption of microwave energy by water molecules in the interior of the berries results in rapid evaporation, causing partial puffing. Thus, the moisture diffusivity during sun-drying may increase considerably, due to increased porosity of the treated grapes (Marousis et al., 1991). A similar effect was reported by Tulasidas et al (1993) and Raghavan et al (1994).

Higher sun-drying rates of alkali-dipped grapes were obtained by increasing the microwave treatment time, e.g. from 0.5 to 2 min at 325 W (Fig. 3). Initially, higher drying rates were observed with increasing power output, but all drying curves tended to end at about the same time. The observed initial acceleration of drying may be caused by an opening of the physical structure allowing rapid evaporation and transport of water. However, all microwave-treated samples tended to dry slowly at the last stages of sun-drying, presumably due to collapse (shrinkage) of the raisin structure. The water diffusivity in grapes increased with microwave treatment, especially at intermediate moisture contents (Raghavan et al., 1994). Application of microwaves to fresh (undipped) grapes was not successful in our experiments, since the berries bulged and exploded, due to the low water permeability of the grape skin. The bulged and exploded berries gave a dried product that was completely different from the familiar shrivelled raisins. A combination of

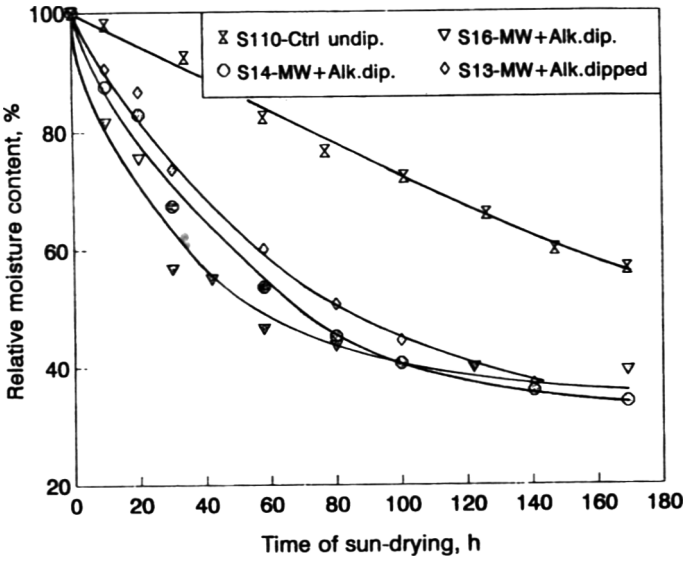

Fig. 3—Effect of microwave-treatment time on the sun-drying rate of Sultana grapes: S13: 325 W (M), 0.5 min. S14: 325 W (M), 1 min. S16: 325 W (M), 2 min.

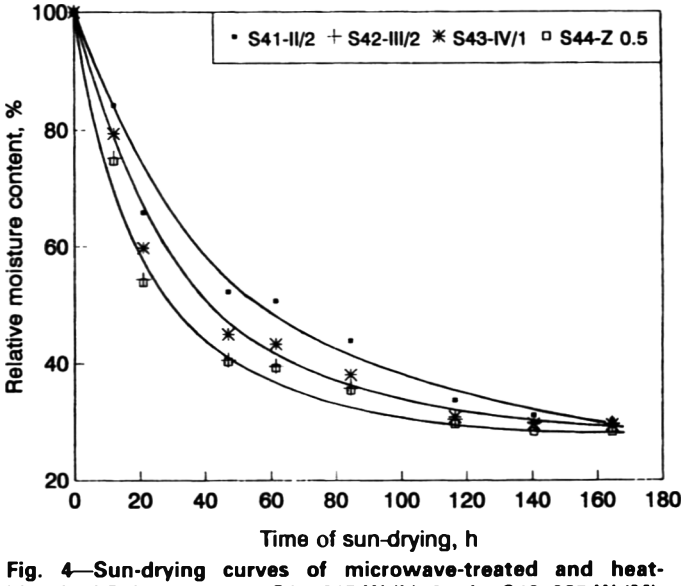

Fig. 4—Sun-drying curves of microwave-treated and heat-blanched Sultana grapes: S41: 215 W (L), 2 min. S42: 325 W (M), 2 min. S43: 430 W (H), 1 min. S44: Blanched at 100°C (Z), 0.5 min.

alkali dipping and short-time microwave treatment appears promising process for reducing sun-drying time.

Thermal blanching (0.5 min in boiling water) resulted in a significant reduction of sun-drying time, an effect similar to microwave pre treatment (samples S41–S44, Fig. 4). A combination of blanching and microwave pre treatment had the same effect on sun-drying as blanching or microwaves alone. Little improvement of sun-drying rate was observed by a combined treatment of alkali dipping, thermal blanching and microwave treatment.

Quality of pre-treated raisins

In addition to improvement of the drying process, the pre treatment of grapes should not damage the quality of dried products. The basic quality characteristics of sun-dried raisins are color, size, appearance and water activity. The first three properties are used by inspectors for grading sun-dried products.

The three color parameters (L, a, b) of experimental raisins, (Table 3) were compared as well as the ratio L/(a/b) and its functions which has been used as quality indicators for raisins and other fruit products (Thai, 1993). This factor correlated well with visual observations, since it takes into consideration all color parameters. The color parameter (L) of the sun-dried raisins increased significantly in the microwave treated sample (S42), meaning that the product was lighter (less brown) than untreated samples (S48). Higher positive values of (b) correspond to more redness, while higher positive values of (a) signify more yellowness. The highest value of the factor L/(a/b) = 48 was observed in the microwave-treated samples S42, S43 (treatment m for 2 min). This factor was 23 in the commercial product and >50 in commercial raisins treated with sulfur dioxide (bleached raisins).

In addition to the data shown (Table 3) the color of four more samples (S13, S14, S16 and S110 of Table 1) was determined with a Hunter Lab colorimeter. Color readings were similar to data obtained with the Minolta colorimeter (Table 3). The appearance of dried raisins, by visual inspection and color photography, showed that the microwave-treated product had a similar appearance to commercial products.

Moisture sorption isotherms of the raisins were not affected by microwave pre-treatments (Fig. 5). The similarity of water activities of experimental samples indicated that little chemical or physicochemical changes were brought about by these pre treatments. No change in water activity indicated that the treated

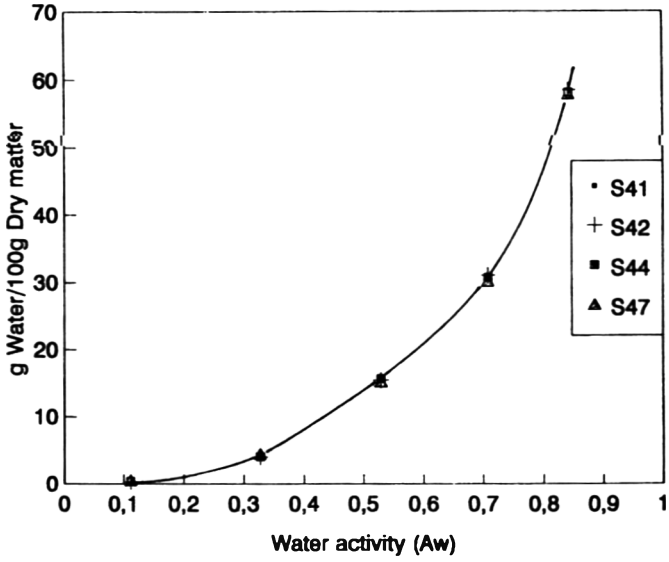

Fig. 5—Water sorption isotherms of microwave-treated and heat-blanched raisins: S41: 215 W (L), 2 min. S42: 325 W (M), 2 min. S44: Blanched at 100°C, 0.5 min. S47: Commercial raisins.

Table 3—Colorimetric parameters of experimental raisins

Sample no	a	b	L	L/(a/b)
S41	7.13	10.11	22.09	31.32
S42	8.35	14.26	28.18	48.13
S43	5.72	9.20	21.14	34.00
S44	7.30	10.42	24.30	34.69
S45	7.07	9.25	24.45	31.90
S46	6.98	9.21	23.24	30.64
S47	5.86	8.63	21.98	32.37
S48	5.60	8.41	21.70	32.59

product could be stored, handled and utilized the same way as commercial raisins.

The increase of lightness (L) of the microwave-treated raisins was evidently the result of the higher drying rate during sun-drying, and/or shorter drying time which resulted in less exposure to the sun. Prolonged drying time and high temperatures are known to increase browning (Gee, 1980). More severe treatment by a combination of thermal blanching and microwave treatment reduced the color of the product (S46), presumably due to increased nonenzymatic browning.

The improvement of color of the microwave-treated raisins may be also due to partial inactivation of enzymes, e.g. polyphenoloxidase (enzymatic browning) in the early stages of drying. Browning enzymes are heat sensitive, and absorption of microwave energy causes rapid heating of food materials. Additionally, non enzymatic browning may have been reduced at the last stages of drying by the high drying rates of the treated grapes. The improvement of drying rate and quality of sun-dried raisins by microwaves is a good incentive for exploring further the feasibility of commercial applications of the process.

REFERENCES

Anonymous, 1989. Microwave food processing. Food Technol. 43(1): 117–126.
Gee, M. 1980. Some flavor and color changes during low temperature dehydration of grapes. J. Food Sci. 45: 146–147.
Giese. J. 1992. Advances in microwave food processing. Food Technol. 46(9): 118–123.
Huxsoll, C.C. and Morgan, A.E. 1968. Microwave dehydration of potatoes and apples. Food Technol. 22: 705–710.
Marousis, S.N., Karathanos, V.T., and Saravacos, G.D. 1991. Effect of physical structure of starch materials on water diffusivity. J. Food Proc. & Pres. 15: 183–195.
Owusu-Ansah, Y.J. 1991. Advances in microwave drying of foods and food ingredients. Can. Inst. Food Sci. Technol. J. 24(3–4): AT 102–197.
Raghavan, G.S.V.. Tulasidas, T.N., Sablani, S.S., and Ramaswamy, H.S. 1994. Concentration dependent moisture diffusivity in drying of shrinkable commodities. In *Drying 94*, V. Rudolph and R.B. Keey (Ed.), Vol. A, p. 277–284, Univ. of Queensland, Brisbane, Australia.
Raouzeos, G.S. and Saravacos G.D. 1986. Solar drying of raisins. Drying Technol. 4(4): 643–649.
Rosenberg, U. and Bogl, W. 1987. Microwave thawing, drying and baking in the food industry. Food Technol. 41(6): 85–91.
Saravacos, G.D. 1986. Mass transfer properties of foods. In *Engineering Properties of Foods*, M.A. Rao and S.S.H. Rizvi (Ed.), p. 89–132, Marcel Dekker, New York.
Saravacos, G.D. and Raouzeos, G.S. 1986. Diffusivity of water in air-drying of raisins. In *Drying '86'*, Vol. 2, A.S. Mujumdar (Ed.), 487–491, Hemisphere Publ., New York.
Saravacos, G.D., Marousis, S.N., and Raouzeos, G.S. 1988. Effect of ethyl oleate on the rate of air-drying of foods. J. Food Eng. 7: 263.
Schiffmann, R.F. 1987. Microwave and dielectric drying. In *Handbook of Industrial Drying*, A.S. Mujumdar (Ed.), p. 327–356, Marcel Dekker, New York.
Schiffmann, R.F. 1992. Microwave processing in the U.S. food processing industry. Food Technol. 46(12): 50–52.
Somogyi, J.P. and Luh, B.S. 1896. Dehydration of fruits. In *Commercial Fruit Processing*, 2nd ed., J.G. Woodroof and B.S. Luh (Ed.), p. 353–405. Van Nostrand Reinhold New York.
Thai, C.N. 1993. Modeling quality characteristics. In *Postharvest Handling--A Systems Approach*, R.L. Shewfelt and S.E. Prussia (Ed.), p. 167–185, Academic Press, New York.
Tulasidas, T.N., Raghavan, G.S.V., and Norris, E.R. 1993. Microwave and covective drying of grapes. Trans. A.S.A.E. 36(6): 1861–1865.
Wolf, W., Spiess, W.E.L., Jung, G., Weisser, H., Bizot, H., and Duckworth, R.B. 1984. The water vapor sorption isotherms of micro crystalline cellulose (MCC) and of purified potato starch: results of a collaborative study. J. Food Eng. 3: 51–72.

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*QUALITY OF DEHYDRATED CARROTS. . .*From page 326

Federal Register (proposed rules). 1988. Department of Health and Human Services. Food & Drug Administration, Part IV (December 19), p. 51084. Washington, DC.
Feiberg, B., Schwimmer, S., Reeva, R., and Juily, M. 1964. *Food Dehydration*, Vol II. The AVI Publishing Co., Inc. Westport, CT.
Inoue, M., Hayatsu, H., and Tanooka, H. 1972. Concentration effect of bisulfite on the inactivation of transforming activity of DNA. Chem. Biol. Interactions 5: 85–95.
Khachik, F., and Beecher, G.R. 1987. Application of a c-45-β-carotene as an internal standard for the quantification of carotenoid in yellow/orange vegetables by liquid chromatography. J. Agric. Food Chem. 35: 732–738.
Mudahar, G.S. 1989. Process optimization and physicochemical investigation of high quality processed vegetables. Ph.D. dissertation. Univ. of Georgia, Athens, GA.
Nutting, M.D., Neumann, H.J., and Wagner, J.R. 1970. Effect of processing variables on the stability of beta-carotenes and xanthophylls of dehydrated parsley. J. Sci. Food. Agric. 21(4): 197–202.
SAS Institute Inc. 1990. *SAS/STAT® User's Guide, Version 6.03 Edition*. SAS Institute Inc., Cary, NC.
Taylor, S.L., Higley, N.A., and Bush, R.K. 1986. Sulfites in foods: uses, analytical methods, residues, fate, exposure assessment, metabolism, toxicity, and hypersensitivity. Adv. Food Res. 30: 1–76.
Tomkins, R.G., Mapson, L.W., Allen, R.J.L., Wager, H.G., and Barker, J. 1944. The drying of vegetables III. The storage of dried vegetables. J. Soc. Chem. Ind. 63: 225–231.
Weier, T.E., and Stocking, C.R. 1949. The influence of selected antioxidants on dehydrated carrots. J. Agric. Res. 78: 503–515.

Ms received 8/21/94; revised 10/27/94; accepted 12/5/94.

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North Dakota Agricultural Experiment Station Journal Article No. 2229.

*POTATO ANTIOXIDANT AND PHENOLIC CONTENT. . .*From page 343

Munshi, C.B. and Mondy, N.I. 1989. Ascorbic acid and protein content of potatoes in relation to tuber anatomy. J. Food Sci. 54: 220–221.
Neuberger, A. and Sanger, F. 1942. The nitrogen of the potato. Biochem. J. 36: 662–671.
Niki, E. 1991. Action of ascorbic acid as a scavenger of active and stable oxygen radicals. Am. J. Nutr. 54: 1119S–1124S.
Niki, E., Tsuchiya, J., Tanimura, R., and Kamiya, Y. 1982. Regeneration of vitamin E from a-chromanoxy radical by glutathone and vitamin C. Chem. Lett. 789–792.
Park, W.D. 1983. Tuber proteins of potato - a new and surprising molecular system. Plant Mol. Biol. Rep. 1: 61–66.
Pratt, D.E. and Watts, B.M. 1964. The antioxidant activity of vegetable extracts I. Flavone aglycones. J. Food Sci. 29: 27–33.
Pratt, D.E. 1972. Water soluble antioxidant activity in soybeans. J. Food Sci. 37: 322–323.
Reeve, R.M., Hautala, E., and Weaver, M.L. 1969. Anatomy and compositional variation within potatoes II. Phenolics, enzymes and other minor components. Amer. Potato J. 46: 374–386.
Rodriguez de Sotillo R., Hadley, M., and Holm, E.T. 1993. Extraction, characterization and antioxidant activity of phenolics in potato peel. Abstract No. 539, presented at the 53rd Annual Meeting of Inst. of Food Technologists, Chicago, IL, July 10-14. SAS Institute, Inc. 1986.
Storey, R.M.J. and Davies, H.V. 1992. Tuber quality. In *The Potato Crop*, P. Harris (Ed.), p. 509–510. Chapman & Hall, London, U.K.
Swain, T. and Hillis, W.E. 1959. The phenolic constituents of *Prunus domestica* L.: The quantitative analysis of phenolic constituents. J. Sci. Food Agric. 10: 63–81.
Taga, M.S., Miller, E.E., and Pratt, D.E. 1984. Chia seed as a source of natural lipid antioxidants. JAOCS 61: 928–931.
Thomas, P. and Joshi, M.R. 1977. Prevention of after-cooking darkening of irradiated potatoes. Potato Res. 20: 77–84.
Tripathi, R.K. and Verma, M.N. 1975. Phenolic compounds and polyphenol oxidase activity in relation to resistance in potatoes against bacterial soft rot. Indian J. Exp. Biol. 13: 414–416.

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Sorption Changes Induced by Osmotic Preconcentration of Apple Slices in Different Osmotic Media

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ABSTRACT

Changes in sorption of dehydrated apple slices induced by osmotic preconcentration (OP) in two different osmotic media were studied. A 55% sucrose solution and a 55% solution of 38 DE Corn Syrup Solids (CSS) were used. Sorption isotherms for osmo-vacuum and vacuum dehydrated product were established and fit to the Guggenheim-Anderson-de Boer (GAB) model. Sorption isotherms of both dehydration treatments shifted with respect to control isotherms. OP in sucrose solution resulted in slight reduction of total sugars and gave the most pronounced isotherm shift, while OP in CSS solution resulted in extensive losses of sugars and a lesser isotherm shift. OP resulted in a product with less sugars than the conventionally dried product, especially when CSS were used.

Key Words: apple slices, osmotic concentration, sorption changes, osmotic media

INTRODUCTION

OSMOTIC PRECONCENTRATION (OP) is a method of partial dehydration by immersing a food material in a highly concentrated solution. It is a minimal process, carried out at relatively low process temperatures (usually <50°C) without phase change. OP has attracted much research interest and demand for commercial applications has grown (Lerici et al., 1988; Nanjundaswamy and Madhakrishniak, 1989). OP provides advantages over conventional dehydration processes, including flavor and aroma retention (Ponting, 1973) and substantial energy savings (Lenart and Lewicki, 1988; Collignan et al., 1992). Furthermore, it provides the potential to freeze/thaw texture-sensitive fruits and vegetables (i.e. melon, strawberries, cucumbers) and constitutes a promising process for freezing-preservation of such products (Lazarides, 1994a).

Developments in OP have been reviewed by Raoult-Wack et al. (1992) and Lazarides (1994b). Solute uptake is sometimes considered a major deficiency of OP as it modifies product composition and may alter natural nutrient profiles. Leaching of product solutes (sugars, acids, minerals, vitamins) into the medium, may also affect the sensory and nutritional characteristics, although it is considered quantitatively negligible (Dixon and Jen, 1977).

Solute uptake also affects product rehydration (hygroscopicity), which for osmotically dried fruit is lower than the untreated in both rate and extent (Ponting et al., 1966; Lerici et al., 1977). This is due to the lower rehydration of sugar in the product, compared to natural tissues. The longer the osmosis time, the lower was the rehydration rate and extent of osmo-convection in dried carrots (Lenart, 1991).

Knowledge of sorption characteristics is essential for designing complimentary processes (i.e. convection, vacuum or freeze dehydration), assuring proper rehydration characteristics and for determination of packaging requirements to provide a desired shelf life. Despite the large volume of work on OP, publications on sorption characteristics of osmotically dehydrated products are rather limited (e.g. Adambounou and Castaigne, 1983; Lerici et al., 1985; Lenart, 1991).

Our main objective was to examine possible changes in sorption characteristics of osmotically preconcentrated apple slices (a model fruit) as related to different patterns of solute exchange caused by the use of different osmotic media. The effects of the most extensively used osmotic solute (sucrose) were compared with corn syrup solids (Lazarides et al., 1994).

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MATERIALS & METHODS

Osmotic preconcentration—Vacuum dehydration

Apples (var. Granny Smith) were used as a model fruit, since they provide fairly homogeneous flesh structure and convenience in obtaining standardized (in size and shape) samples. The specific cultivar was selected for its good post harvest stability in terms of maturity and hardness. Sample preparation and experimental procedure were described in detail in a previous publication (Lazarides et al., 1994). Sugar and corn syrup solids (38 DE) were used as osmotic solutes. The osmotic solution concentration was 55%, the solution/product ratio was 30:1 and the osmotic process was continued for up to 5 hr at 50°C under continuous gentle stirring. Following preconcentration, apple slices were vacuum dehydrated to constant weight at 60°C and 6.7 kPa.

Sorption isotherms

Sorption isotherm data were produced through moisture equilibration of dehydrated samples over saturated salt solutions of known relative humidities (static gravimetric method) at constant temperature (30°C). Sorption isotherms were developed according to the standard procedure described in the COST 90 project of EEC (Spiess and Wolf, 1983).

Ten 1-L glass jars (hygrostats) were used, each containing a saturated salt solution selected to produce a specific relative humidity (Greenspan, 1977). The following salts were used to give corresponding relative humidities (in parenthesis): LiCl (0.113), $K(CH_3COO)$ (0.216), $MgCl_2$ (0.324), K_2CO_3 (0.432), KNO_3 (0.472), $Mg(NO_3)_2$ (0.514), $NaNO_2$ (0.633), NaCl (0.751), $(NH_4)_2SO_4$ (0.806) and KCl (0.836).

Equilibration was reached within 15 days. During that period the hygrostats were kept immersed in a custom made, well insulated and covered water bath with good water circulation and close temperature control (+/− 0.2°C). A small amount of thymol was used to prevent fungal growth in the higher relative humidity hygrostats. Equilibrium moisture content was determined by vacuum dehydration according to AOAC (1990) method number 934.06. Triplicate samples were used with each hygrostat and equilibration was run with three different sets of samples from three different osmotic preconcentration runs.

Isotherm modelling

Sorption isotherm data were fitted to the GAB (Guggenheim-Anderson-de Boer) equation. Comparative analysis has established that this mathematical model best describes sorption isotherms of most foods for the widest water activity range (Van den Berg, 1985). Each of the three GAB constants has a specific physical meaning.

The GAB equation can be written in the following form:

$$m = m_0 C k a_w/[(1 - k a_w)(1 - k a_w + C k a_w)] \quad (1)$$

where: m is the equilibrium moisture content in g water/g dry solids, m_0 is the monolayer moisture content (in g water/g dry solids), C is the Guggenheim constant related to heat of sorption for the first layer, k is a constant related to the heat of sorption for multilayer water, and a_w is water activity.

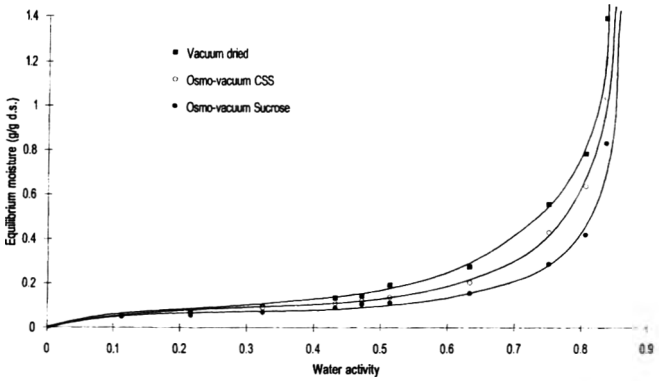

Fig. 1—Sorption isotherms for vacuum and osmo-vacuum dehydrated apple slices using two different osmotic media.

The GAB equation constants were calculated using the steepest ascent optimization technique in a BET/GAB computer program (developed by Kathy Nelson and Theodore P. Labuza, Food Science Dept., Univ. of Minnesota). Besides the three equation constants, heat of sorption (Q_s), % standard error and the mean relative deviation modulus (P) were also calculated. P is a criterion used to evaluate goodness of fit and is described by the following equation (Lomauro et al., 1985):

$$P = (100/n) \Sigma_{i=1} |(m_i - m_{pi})|/m_i \quad (2)$$

where: m_i is the experimental (observed) equilibrium moisture content, m_{pi} is the predicted equilibrium moisture content, and n is the number of observations.

P-values <5.0 indicate an excellent fit, while values >10.0 are indicative of a poor fit.

Sugars

Fructose, glucose and sucrose determination was carried out by HPLC according to the procedure described by Wilson et al. (1981). A Waters Chromatograph was used consisting of a Model 510 solvent delivery system, a Waters μBondapak Carbohydrate Analysis column (3.9 × 300 mm), a Model 410 Differential Refractometer detector and a Rheodyne 7125 injector. Data were acquired and analyzed using a Waters Maxima 820 work station. The mobile phase was 80% acetonitrile in water and the flow rate was 1.8 ML/min. Triplicate samples were run for each treatment.

RESULTS & DISCUSSION

SORPTION ISOTHERMS for the three experimental treatments were compared (Fig. 1). All isotherms had a shape characteristic of hygroscopic materials, with low equilibrium moisture contents at low or moderate a_w and sharply increased moistures at higher a_w. Sorption isotherm constants and regression parameters (Table 1) from vacuum dehydrated apples gave a satisfactory fit to the GAB model (P = 6.4%), while those for osmo-vacuum dehydration in sucrose and CSS solution gave a poor (P = 14.5%) or mediocre fit (P = 9.7%). These results confirmed generally those reported by other researchers (Lomauro et al., 1985). Note that both osmotic media resulted in shifting and distortion of the sorption isotherm with respect to the control, strongly affecting the degree of fit. OP in sucrose solution gave the widest shift and the largest distortion (largest P-value). In terms of product rehydration, the observed isotherm shift indicated better rehydration characteristics for the control compared to both OP treatments, with sucrose solution giving lowest product rehydration.

Differences among sorption isotherms are reflected on certain GAB constants. The monolayer moisture content (m_o) for the vacuum dehydrated product was higher than that of either OP treatment and well within the range of values reported by others (Lomauro et al., 1985). Following OP, the monolayer moisture content was decreased by ca. 35% for sucrose solution and 20% for CSS solution, compared to that of the control.

The remarkable feature of all three apple isotherms was the fact that the sorption term became significant at very low a_w (below 3.0), which also confirmed reports of other workers (Rotstein and Cornish, 1978). According to Jeffrey (1982), at very low moisture contents internal hydrogen bonding occurs in carbohydrate molecules. Such molecules are then organized in a crystal lattice by including as many hydrogen bonds as possible, providing to the outside a hydrophobic interface.

A 34% and 23% increase in heat of sorption (Q_s) occured for the two osmotic treatments, compared to the control. This was naturally translated into higher water binding forces and respectively more energy required to remove water during dehydration. At the same water content, sucrose-impregnated products dried slower than fresh ones (Collignan et al., 1992).

As shown (Fig. 1), a definite isotherm shift occured between the two OP treatments and the control. This shift was probably due to compositional changes occurring during osmotic preconcentration in different osmotic media. Sugar profile changes induced by the osmotic preconcentration treatments were followed (Table 2).

During OP in sucrose, the product showed a high loss of fructose (ca. 1/3 the initial content) and this loss occured within the first hour of preconcentration (Table 2). When CSS were used as osmotic solute, only half of that loss was observed within the first hour. As OP proceeded, however, drastically higher levels of fructose loss were attained (43% vs. 15%). Glucose losses followed a similar pattern, although with greater differences between 1 and 5 hr of OP for both osmotic media. 72% of glucose losses were observed within the first hour, while respective losses for CSS were 20% of total (5 hr) losses.

A totally different behavior of the two osmotic media occured with respect to sucrose losses. OP in sucrose solution resulted in extensive sucrose uptake (up to 150% the original content with 85% of the total uptake occurring within the first hour). However, OP in CSS resulted in massive losses of sucrose, leaving the final product with only 15% its initial sucrose content after 5 hr.

Overall, OP in sucrose solution resulted in high losses of fructose and glucose, and extensive increase of sucrose. Major part of these changes (57% for fructose, 72% for glucose and 85%

Table 1—GAB constants and regression parameters for sorption isotherms of vacuum and osmo-vacuum dehydrated apple slices

Parameter	Vacuum dehydrated (control)	Osmo-vacuum dehydrated	
		Sucrose soln	CSS soln
m_O, g water/g solids	0.0775	0.0514	0.0630
C	9.31	19.62	15.40
k	1.13	1.14	1.14
Q_S, cal/mole	1321	1763	1619
Std error, %	0.3389	1.0486	1.6192
P value, %	6.4	14.5	9.7

Table 2—Sugar profile changes induced by osmotic preconcentration of apple slices in different osmotic media

Sugar	Sugar concentration, g/g dry solids				
	Vacuum dehydrated (Control)	Sucrose		Corn syrup solids	
		Preconcentration time			
		1 hr	5 hr	1 hr	5 hr
Fructose	0.3350*	0.2190	0.2124	0.2834	0.1896
(+/− s.d.)	(0.0035)	(0.0105)	(0.0025)	(0.0018)	(0.0026)
% change		−34.6%	−36.6%	−15.4%	−43.4%
Glucose	0.3543	0.2520	0.2126	0.3144	0.1575
(+/− s.d.)	(0.0220)	(0.0089)	(0.0024)	(0.0042)	(0.0014)
% change		−28.9%	−40.0%	−11.3%	−55.6%
Sucrose	0.1481	0.3370	0.3684	0.0939	0.0235
(+/− s.d.)	(0.0011)	(0.0148)	(0.0041)	(0.0055)	(0.0047)
% change		+127.6%	+148.7%	−36.6%	−84.1%
Total	0.8374	0.8080	0.7934	0.6917	0.3706
% change		−3.5%	−5.3%	−17.4%	−55.8%

* Means of triplicate samples (+/− standard deviation).

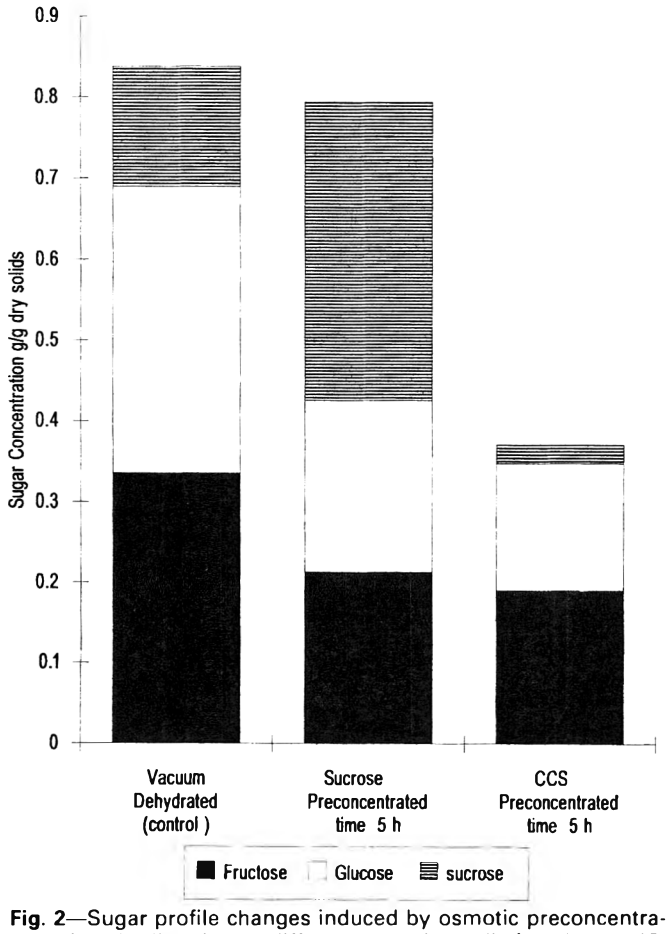

Fig. 2—Sugar profile changes induced by osmotic preconcentration of apple slices in two different osmotic media for 5 hr at 50°C.

for sucrose) occured within the first hour of OP. On the other hand, OP in CSS resulted in high losses (43 to 84% the initial content) for all 3 sugars with much lower losses within the first hour (20 to 44% of total losses).

In terms of total solids exchange, OP in sucrose solution gave a small net loss of solids (3.5% within the first hour, 5.3% in 5 hr). OP in CSS resulted in extensive net loss of solids, reaching 17% within the first hour and exceeding 55% the original amount in 5 hr. As a result, the final CSS product had ≃ half the total sugar content of the final product which was treated in sucrose solution. Compared to the sucrose product, the CSS product had ≃ 10, 25 and 95% less fructose, glucose and sucrose, respectively. Such selective leaching of sugars resulted in a sugar profile which could be desirable to consumers as reduced calorie products.

Total sugar levels and sugar profiles for the 3 treatments were graphically compared (Fig. 2). Differences among treatments in total sugars and sugar composition were reflected in sorption effects. Different sugars exhibit different water sorption properties; i.e. the ability of amorphous glucose to sorb a given amount of water is greater than that for crystalline sucrose (Smith et al., 1981). This may be due to differences in binding site availability and bond energies for different structures. At low moisture levels water is strongly bound to active sites and does not enhance solution or plasticizing processes (Van den Berg and Bruin, 1981). At higher levels, however, a combination of actions occurs resulting in a sharp increase in adsorptive capacity, as evidenced in all three experimental isotherms (Fig. 1). Such actions include solution, new site creation (by swelling), plasticizing and water-water adsorption. Minor sugars and other soluble constituents probably dissolve before the main sugars. These observations have important practical effects, especially in dry product rehydration and rehydrated product stability.

CONCLUSIONS

OSMOTIC PRECONCENTRATION (OP) of apple slices in sugar solutions was followed by massive exchange of sugars between products and the osmotic medium. This exchange strongly related to the osmotic solution and was faster within the first hour of OP. OP in solution of CSS resulted in massive leaching (loss) of sucrose and limited losses of fructose and glucose, providing a final product with half its initial sugar content. OP in sucrose solution, however, provided a more stable total sugar balance, due to extensive uptake of sucrose counteracting most losses in fructose and glucose. Early interruption of OP had much greater impact on solids exchange after use of CSS than after use of sucrose. OP in CSS favored retention of fructose and glucose, and diminished loss of sucrose and total solids. Sugar exchange during OP resulted in highly modified sugar profiles of final products with significant effects on sorption. An isotherm shift occured in the vacuum dried product, caused by differences in binding properties of the three main sugars. The practical consequence was different product responses to dehydration and rehydration processes.

REFERENCES

Adambounou, T.L. and Castaigne, F. 1983. Deshydratation par osmose de bananes et determination de courbes de sorption isotherme. Lebensm.-Wiss. u.-Technol. 16: 230–234.

AOAC, 1990. *Official Methods of Analysis*, 15th ed. Association of Official Analytical Chemists, Washington, DC.

Collignan, A., Raoult-Wack, A.L. and Themelin, A. 1992. Energy study of food processing by osmotic dehydration and air drying. Ag. Engineering J. 1(3): 125–135.

Dixon, G.M. and Jen, J.J. 1977. Changes of sugars and acids of osmovac-dried apple slices. J. Food Sci. 42: 1126–1127.

Greenspan, L. 1977. Humidity fixed points of binary saturated aqueous solutions. J. Res. of NBS. A. Physics & Chem. 81A 1: 89–96.

Jeffrey, G.A. 1982. Hydrogen bonding in amino-acids and carbohydrates. In *Molecular Structure and Biological Activity*, J.F. Griffin and W.L. Duax (Ed.), p. 135. Elsevier Science Publishing Co., Amsterdam.

Lazarides, H.N. 1994a. Osmotic preconcentration as a tool in freeze preservation of fruits and vegetables. Proceedings of the Second International Seminar on "Osmotic Preconcentration of Fruits and Vegetables," April 18–19, Warsaw, Poland.

Lazarides, H.N. 1994b. Osmotic dehydration: Developments and prospects. In *Minimal Processing of Foods: Process Optimization* , R.P. Singh and F. Oliveira (Ed.), p. 73–85. CRC Press, Boca Raton, FL. In press.

Lazarides, H.N., Katsanidis, E. and Nickolaidis, A. 1994. Mass transfer kinetics during osmotic preconcentration aiming at minimal solid uptake. J. Food Eng. In press.

Lenart, A. 1991. Effect of saccharose on water sorption and rehydration of dried carrot. In *Drying '91*, A.S. Mujumdar and I. Filkova (Ed.), p. 489. Elsevier Science Publ., Amsterdam.

Lenart, A. and Lewicki, P.P. 1988. Energy consumption during osmotic and convective drying of plant tissue. Acta Alimentaria Polonica, 14 (1): 65–72.

Lerici, C.R., Mastrocola, D., Sensidoni, A., and Dalla Rosa, M. 1988. Osmotic concentration in food processing. In *Preconcentration and Drying of Food Materials*, S. Bruin (Ed.), p. 123. Elsevier Science Publishers B.V., Amsterdam.

Lerici, C.R., Pinnavaia, G., Dalla Rosa, M., and Bartolucci, L. 1985. Osmotic dehydration of fruit: Influence of osmotic agents on drying behaviour and product quality. J. Food Sci. 50: 1217–1219, 1226.

Lerici, C.R., Pepe, M., and Pinnavaia, G. 1977. La disidratazione della frutta mediante osmosi diretta. I. Risultati di esperienze effettuate in laboratorio, Industria Conserve 52(2): 125–129.

Lomauro, C.J., Bakshi, A.S., and Labuza, T.P. 1985. Evaluation of food moisture sorption isotherm equations. Part I: Fruit, vegetable and meat products. Lebensm.-Wiss. u. Technol. 18: 111–117.

Nanjundaswamy, A.M., and Madhakrishniak, S.G. 1989. Advances in dehydration processes for fruits, vegetables and their products. In *Trends in Food Science and Technology*, M.R. Ragavenfra Rao et al. (Ed.), p. 369. India.

Ponting, J.D. 1973. Osmotic dehydration of fruits—Recent modifications and applications. Process Biochem. 8: 18–20.

Ponting, J.D., Watters, G.G., Forrey, R.R., Jackson, R. and Stanley, W.L. 1966. Osmotic dehydration of fruits, Food Technol. 29(10): 125–128.

Raoult-Wack, A.L., Lenart, A., and Guilbert, S. 1992. Recent advances in dewatering through immersion in concentrated solutions ("Osmotic dehydration"). In *Drying of Solids*, A.S. Mujumdar (Ed.), p. 21. Elsevier Science Publ. B.V.

Rotstein, E. and Cornish, A.R.H. 1978. Prediction of the sorption equilibrium relationship for the drying of foodstuffs. AIChE J. 24: 956.

Smith, D.S., Mannheim, C.H., and Gilbert, S.G. 1981. Water sorption isotherms of sucrose and glucose by inverse gas chromatography. J. Food Sci. 46: 1051–1053.

Spiess, W.E.L. and Wolf, W.R. 1983. The results of the COST 90 project on water activity. In *Physical Properties of Foods*, Jowitt et al. (Ed.), p. 65. Applied Science Publishers, London.

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Expression of Anthocyanins in Callus Cultures of Cranberry (*Vaccinium macrocarpon* Ait)

D.L. MADHAVI, M.A.L. SMITH, and M.D. BERBER-JIMÉNEZ

ABSTRACT

Expression of anthocyanins and other flavonoids in callus cultures established from different parts of cranberry plant was investigated and the effect of explant source on the *in vitro* product was determined. Callus cultures were initiated from different parts of the plant in a modified Gamborg's medium with 5.37 μM α-naphthaleneacetic acid, 0.45 μM 2,4-dichlorophenoxyacetic acid, and 2.32 μM kinetin in the dark at 25°C. Callus cultures accumulated anthocyanins only on exposure to light and maximum concentration was observed by day 12. The cultures had lower levels of anthocyanins and only cyanidin 3-galactoside, cyanidin 3-glucoside, and cyanidin 3-arabinoside were identified in all cultures regardless of source of explant. Proanthocyanidin accumulation in cultures was independent of light, and levels were higher than in mature fruit. Exposure to light induced accumulation of flavonols and enhanced activity of phenylalanine ammonia-lyase in the cultures.

Key Words: anthocyanins, cranberry, callus, cyanidin

INTRODUCTION

THE CHARACTERISTIC bright red anthocyanin pigments in cranberries (*Vaccinium macrocarpon* Ait) make them an attractive potential source for natural food colors. Consumer demand for natural products has prompted new research on development of cell culture resources for pigment extraction. *In vitro* culture of highly pigmented genotypes may circumvent seasonal and geographic restrictions of cranberry crop production, and result in more product uniformity (Ilker, 1987; Shuler et al., 1990; Stafford, 1991). Cell cultures also provide effective systems for elucidating the biochemical aspects of secondary product formation, or for exploring the physiological properties of intermediates in biosynthetic pathways. Cell cultures may accumulate different secondary metabolites from those found *in vivo*, or may produce insignificant quantities of some components (Nawa et al., 1993; Mori et al., 1993; Wilson, 1990). In other cases, product yields and/or quality traits may be enhanced through production by tissue culture (Cormier and Do, 1993; Crouch et al., 1993). Manipulation of the physical and chemical microenvironments can influence both the quality and yield of natural products (Callebaut et al., 1990; Do and Cormier, 1991).

Anthocyanin production has been reported in cell cultures of edible materials like grapes (Yamakawa et al., 1983), sweet potato (Nozue et al., 1987), carrot (Ozeki and Komamine, 1985), strawberry (Hong et al., 1989; Mori et al., 1993), and rabbiteye blueberry (Nawa et al., 1993). Cranberry has been cultured *in vitro* for micropropagation (Marcotrigiano and McGlew, 1991) and genetic transformation (Serres et al., 1992), but *in vitro* pigment production has not been reported. Our objective was to compare anthocyanins derived from cranberry fruits *in vivo*, vegetative parts, and callus cultures derived from different parts of the plant. The expression of other flavonoids like flavonols and proanthocyanidins, *in vivo* and *in vitro* were also investigated and the activity of phenylalanine ammonia-lyase, one of the early enzymes in the flavonoid biosynthetic pathway, was determined to help elucidate the anthocyanin biosynthetic potential of the cultures.

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MATERIALS & METHODS

CRANBERRY 'STEVENS' FRUITS were obtained from the Blueberry and Cranberry Research Center, Rutgers University, and stored at −20°C until assayed. Plants of the same genotype were maintained in the greenhouse and as shoot cultures in WPM medium (Lloyd and McCown, 1981) supplemented with 0.98 μM 6-(γ,γ-dimethylallylamino) purine.

Callus initiation and pigment production

Stem segments (1 cm) and leaves from 8 wk old shoot cultures and the mesocarp tissue of mature fruits were used for callus initiation. Explants were placed in the dark at 25°C on a callus induction medium (modified Gamborg's medium, Gamborg et al., 1968) with 8mM NO_3^- as KNO_3, 25 mM NH_4^+ as $(NH_4)_2SO_4$, 200 μM Fe as $FeNa_2EDTA$, 5.37 μM α-naphthaleneacetic acid, 0.45 μM 2,4-dichlorophenoxyacetic acid, 2.32 μM kinetin, 100 mg/L PVP (Sigma Chemical Co., St. Louis, MO), 10% coconut water, 2% sucrose, and 0.7% agar. Subcultures were derived at 3 wk intervals. After the fourth subculture, callus colonies were transferred to pigment production medium containing no coconut water, reduced NO_3^- (2mM) and increased sucrose (5%) and held under a photosynthetic photon flux of 55 μmol m^{-2} s^{-1}. Time course experiments for anthocyanin production were done both at 8mM and 2mM NO_3^- levels in the production medium. Total anthocyanins were estimated by the method of Francis (1982). Results are averages of two replicates.

HPLC analysis of anthocyanins and anthocyanidins

The samples were extracted in 1% (v/v) HCl methanol (1:10 w/v) overnight at 4°C and the extracts after filtration were evaporated to dryness at 30°C with a Buchi rotary evaporator (Switzerland). The residue was dissolved in water and extracted with ethyl acetate (1:3 v/v) three times. The aqueous fraction was evaporated to remove residual ethyl acetate and adsorbed onto an activated OnGuard-RP Sep-Pak cartridge (Dionex, CA). The cartridge was washed with water and anthocyanins were eluted with 0.01% HCl in methanol. The extract was evaporated, redissolved in 10% formic acid, and filtered through a 0.2 μm filter membrane (Phenomenex, CA). HPLC was performed using a Hitachi L-6200A Intelligent Pump (Tokyo, Japan), a Hitachi Diode Array Detector (Tokyo, Japan), and a Rheodyne (Cotiati, CA) 7125 Injector. The column was a YMC-Pack ODS-AM (250 × 4.6 mm, 5 μ) connected to a YMC-Pack S5 120A ODS-AM guard column. Solvents were 10% formic acid (A) and 100% acetonitrile (B) at 1 mL/min. The elution profile was 0–4 min, 10–14% B in A; 4–10 min, 14–16% B in A; 10–20 min, 16–18% B in A; 20–25 min, 18–30% B in A. Absorbance was monitored at 520 nm. Peak identification was done using authentic standards isolated from the fruit and other known sources.

Anthocyanins from fruit extract and callus cultures were subjected to acid hydrolysis in 2N HCl:methanol (1:1) for 30 min as described by Markham (1982). Anthocyanidins derived from acid hydrolysis were analysed using a Whatman Partisil ODS-3 (250 × 4.6 mm, 5μ) column connected to a YMC-Pack S5 120A ODS-AM guard column. Solvents were 10% formic acid (A) and 100% acetonitrile (B). Separation was obtained by an isocratic elution of 20% B in A at 1 mL/min.

HPLC analysis of other flavonoids

The ethyl acetate-extractable fraction was washed with water, passed through anhydrous sodium sulfate to remove residual moisture and evap-

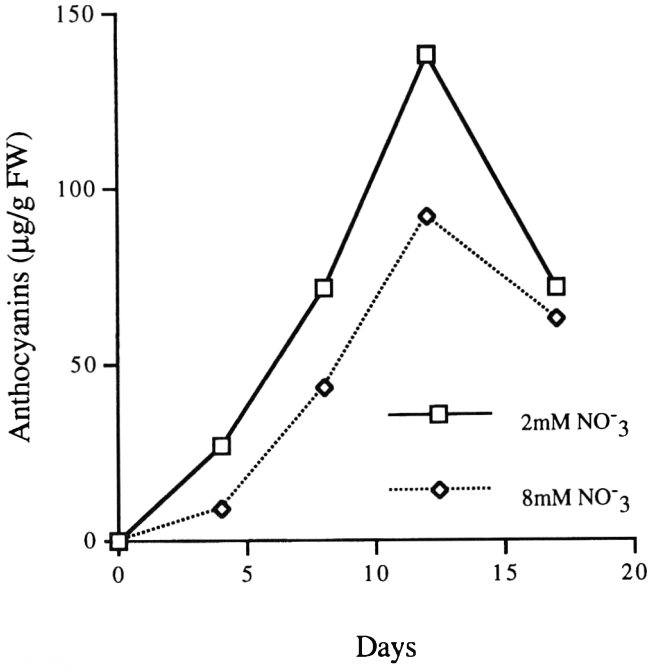

Fig. 1—Time course of anthocyanin formation in stem callus.

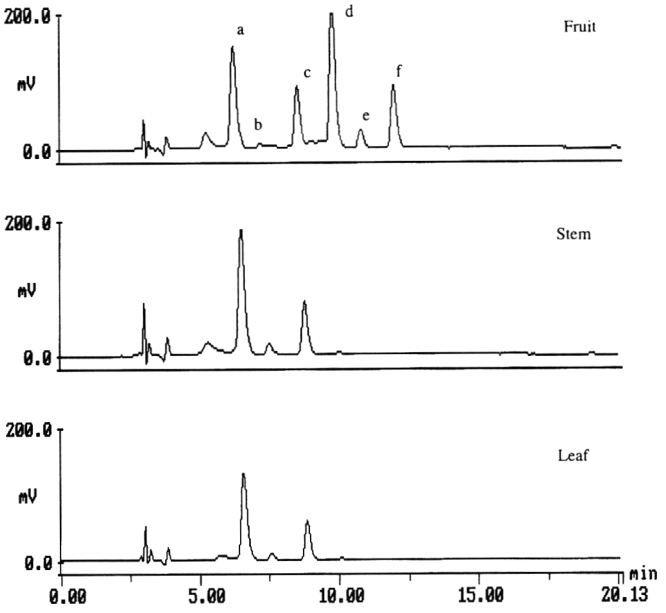

Fig. 2—Reverse phase HPLC profile of anthocyanins in cranberry fruit and vegetative tissues. Peak identification: a. Cyanidin 3-galactoside, b. Cyanidin 3-glucoside, c. Cyanidin 3-arabinoside, d. Peonidin 3-galactoside, e. Peonidin 3-glucoside, f. Peonidin 3-arabinoside

orated to dryness at 30°C *in vacuo*. The residue was dissolved in methanol. Conditions for HPLC analysis were similar to anthocyanin analysis with an altered elution profile: 0–4 min, 10–14% B in A; 4–10 min, 14–18% B in A; 10–20 min, 18–30% B in A; 20–30 min, 30–50% B in A; 30–35 min, 50–100% B in A. Absorbance was monitored at 280 nm.

Proanthocyanidins

Proanthocyanidins were estimated as anthocyanidins by the method of Nawa et al. (1993). Samples were repeatedly extracted in cold (4°C) 1% HCl methanol to remove all pigments. The residue after pigment extraction was suspended in 1% HCl methanol (1:10 w/v), incubated overnight at 50°C then filtered. Absorbance was measured at 535 nm and expressed as absorbance units (A.U.)/g fresh weight (FW). Results are averages of two replicates.

Phenylalanine ammonia-lyase (PAL) activity

Samples were ground in liquid N_2 to a fine powder and suspended in −20°C acetone for 15 min. The precipitate was collected by filtration, washed with acetone and dried at room temperature (25°C). Acetone powders were extracted with 0.1M borate buffer, pH 8.8, containing 20mM 2-mercaptoethanol. 0.5g dry Polyclar AT (ISP Technologies, Inc., Wayne, NJ), and 0.5g dry Amberlite XAD-4 (Sigma Chemical Co., St. Louis, MO). The extract was pressed through cheesecloth and centrifuged cold (4°C) at 10,000 rpm for 20 min. The supernatant was dialyzed overnight (4°C) in 0.1M borate buffer, pH 8.8, containing 20mM 2-mercaptoethanol. The dialyzate was centrifuged cold (4°C) at 10,000 rpm for 10 min and used for assay.

Enzyme activity was measured by the method of Havir and Hanson (1971). The assay mixture contained 0.5 mL of 0.2M borate buffer, pH 8.8, 0.2 mL enzyme solution, and water to a final volume of 2.8 mL. Control assays contained buffer or water in place of enzyme solution or L-phenylalanine. The mixture was incubated at 30°C for 30 min. The reaction was initiated by addition of 0.2 mL of 0.1M L-phenylalanine. The absorbance of the reaction mixtures and blanks was recorded at 280 nm at 15 min intervals using a Beckman DU-65 spectrophotometer (Fullerton, CA) with a temperature controlled auto-6 sampler, at 30°C. One unit of activity was defined as the amount of enzyme catalyzing the formation of 1 µmole cinnamate/min at 30°C. Results are averages of three replicates. Protein was estimated by Bradford's method (Bradford, 1976).

RESULTS

Callus cultures and anthocyanin formation

A yellow colored callus was obtained in the dark in callus induction medium. Modification of Gamborg's medium was necessary to enhance growth and reduce callus culture browning. An increase in NH_4^+ level and a decrease in NO_3^- level with a 3:1 NH_4^+ and NO_3^- ratio was essential to reduce browning and induce friability of the cultures. Addition of coconut water enhanced callus growth. On transferring cultures to light in the production medium, intense pigmentation was observed. Maximum anthocyanin formation was observed by day 12 (Fig. 1) after transferring cultures to light. The level of NO_3^- in the production medium had an effect on anthocyanin concentration. At the 2 mM level, a higher anthocyanin accumulation was observed on all days.

Anthocyanins and anthocyanidins

Reverse phase HPLC chromatograms of the anthocyanins *in vivo* from fruits and from stem and leaves (Fig. 2) showed fruit extract had four major pigments comprised of cyanidin and peonidin 3-galactosides and 3-arabinosides. Cyanidin and peonidin 3-glucosides were the minor pigments. Stems and leaf tissues from the greenhouse and shoot cultures had only cyanidin 3-galactoside, 3-glucoside, and 3-arabinoside, with the glucoside as the minor fraction. The anthocyanin profiles (Fig. 3) showed simple profiles consisting of mainly cyanidin 3-galactoside, 3-glucoside, and 3-arabinoside in all pigmented callus cultures regardless of source of explant. Anthocyanins were not detected in dark grown callus cultures. On acid hydrolysis of the anthocyanins, cyanidin and peonidin were the aglycones in the fruit extract and cyanidin was the only aglycone in the callus cultures (Fig. 4).

Proanthocyanidins

Incubation of the tissue residues in methanol-HCl at 50°C for 18 hr resulted in the release of intense red pigments from all samples with absorbance maximum at 535 nm. The pigments were not extractable by cold methanol-HCl. The A.U./g FW from anthocyanins and anthocyanidins derived from proantho-

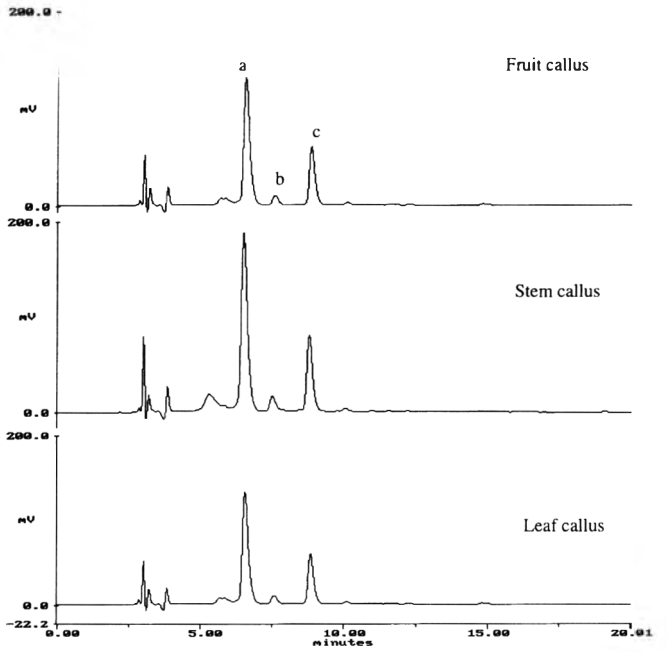

Fig. 3—Reverse phase HPLC profile of anthocyanins in cranberry callus cultures. Peak identification: a. Cyanidin 3-galactoside, b. Cyanidin 3-glucoside, c. Cyanidin 3-arabinoside

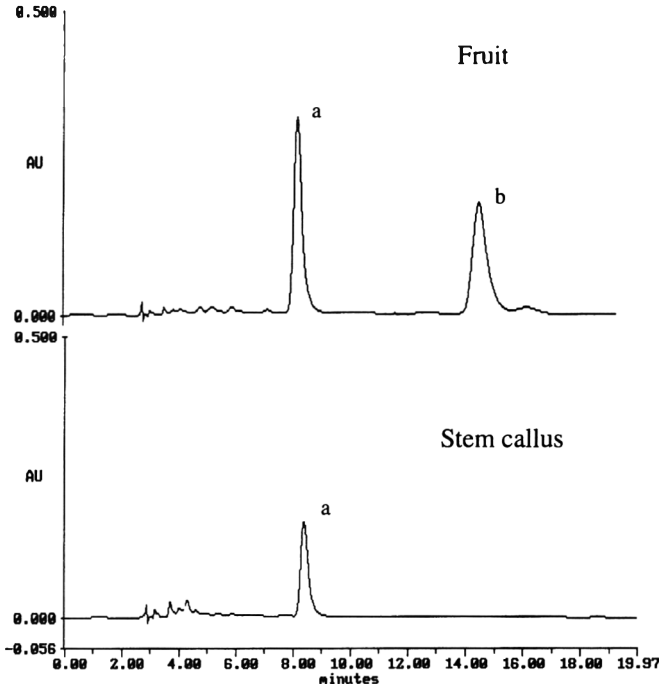

Fig. 4—Reverse phase HPLC profile of anthocyanidins from cranberry fruit (*in vivo*) and stem callus (*in vitro*). Peak identification: a. Cyanidin, b. Peonidin.

cyanidins in vivo and in vitro (Table 1) showed fruit extract had higher absorption due to anthocyanins whereas extracts of vegetative explants had a very high absorption due to proanthocyanidins. Fruit callus culture extracts had a very low level of anthocyanins and a higher proanthocyanidin absorption. Pigmented callus cultures from stems and leaves had a higher anthocyanin level and a lower absorption due to proanthocyanidins. Callus cultures in the dark accumulated proanthocyanidins at comparable levels to cultures under light.

Other flavonoids

Peaks from reverse phase HPLC chromatograms of the ethyl acetate-extractable fractions were classified tentatively into two major groups of compounds based on spectral properties. Group 1 was comprised of compounds absorbing mainly at 270–290 nm, indicative of catechins, flavanones, and those absorbing at 280–330 nm, indicative of cinnamic acids. Group 2 was comprised of compounds absorbing at 280, 350–375 nm, indicative of flavonols and their glycosides (Table 2). Fruit extract had a higher percentage of group 2 compounds whereas vegetative explants had a higher percentage of group 1 compounds. Pigmented callus cultures from vegetative explants were similar to explants. However, pigmented fruit callus culture had a much lower percentage of group 2 compounds in contrast to the fruit. Dark-grown callus cultures (colorless) had mainly group 1 compounds, and flavonols and their derivatives were not detected (Fig. 5).

Phenylalanine ammonia-lyase activity

Sample enzyme activity (Table 3) showed dark-grown callus cultures had a lower activity. A threefold increase in activity was observed on exposure to light. However, all cultures had lower levels of activity compared to explants.

Table 1—Absorbance due to anthocyanins and proanthocyanidins in cranberry *in vivo* and *in vitro*

Source	Anthocyanins (A.U./g FW)	Proantho-cyanidins (A.U./g FW)
Fruit (*in vivo*)	29.62	21.46
Stem (from microcultures)	1.04	82.59
Leaf (from microcultures)	0.61	85.31
Stem Callus (colored)	2.52	27.39
Stem Callus (colorless)	0.112	23.5
Leaf Callus (colorec)	3.31	49.2
Fruit Callus (colored)	2.19	57.31
Fruit Callus (colorless)	0.254	59.5

Table 2—Percentage of total peak area of compounds in ethyl acetate-extractable fraction from cranberry *in vivo* and *in vitro*

Source	Absorbance 270-290 nm[a] 280-320 nm[b]	Absorbance 280, 350-380 nm[c]
Fruit (*in vivo*)	33.7	66.2
Stem (from microcultures)	68.1	31.9
Leaf (from microcultures)	70.1	29.9
Stem Callus (colored)	79.8	20.2
Stem Callus (colorless)	100	—
Leaf Callus (colored)	73.0	27
Fruit Callus (colored)	93.0	7.0
Fruit Callus (colorless)	100	—

[a] Compounds with absorption spectra of catechins, flavanones
[b] Compounds with absorption spectra of cinnamic acids
[c] Compounds with absorption spectra of flavonols and their glycosides

DISCUSSION

OUR MAIN OBJECTIVE was to establish and compare cell cultures from different parts of cranberry plants for their potential to accumulate anthocyanins and other flavonoids and to determine the influence of explant source on composition of in vitro products. Anthocyanins in cranberry fruits are well characterized and our results confirmed previous reports (Hong and Wrolstad, 1990; Fuleki and Francis, 1967). Callus cultures resembled other anthocyanin producing systems in many properties. Callus cultures accumulated anthocyanins only on exposure to light. Anthocyanin level was lower as in other cell culture systems like rabbiteye blueberry (Nawa et al., 1993). Higher sucrose levels and lower nitrate levels enhanced anthocyanin accumulation in parallel with other cell culture systems like grapes (Do and Cormier, 1991; Hirasuna et al., 1991). Previous studies in our lab-

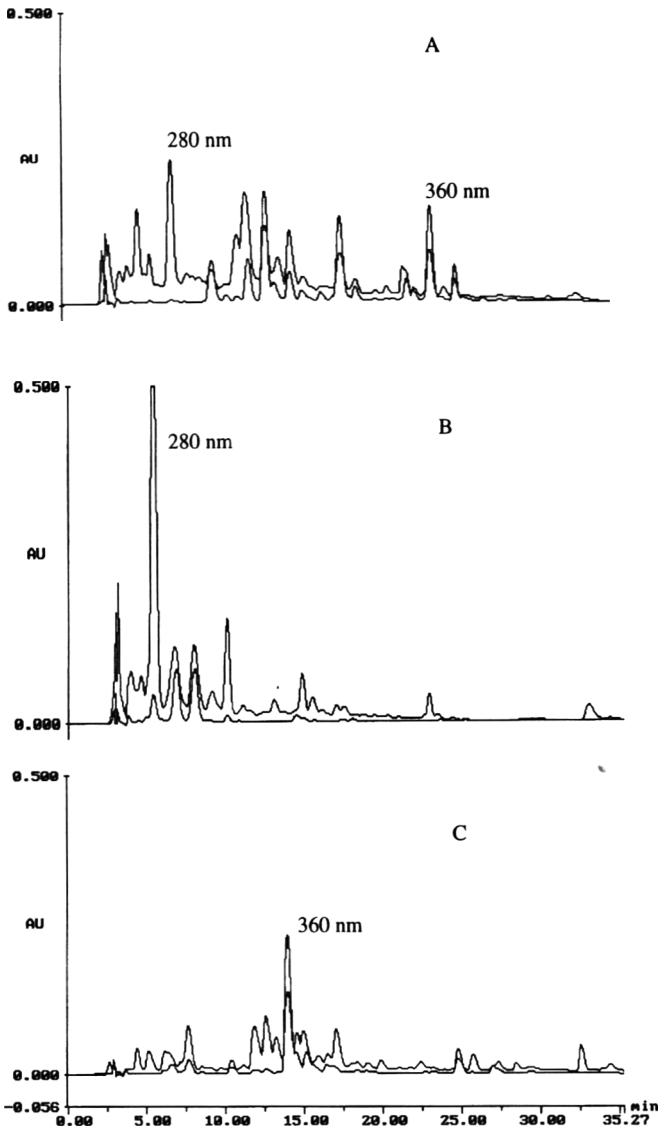

Fig. 5—Reverse phase HPLC profile of the ethyl acetate-extractable fraction. A. Stem, B. Stem Callus (colorless), C. Stem Callus (colored).

Table 3—Phenylalanine ammonia-lyase activity from cranberry *in vivo* and *in vitro*

Source	Units × 10 ^{3}/g FW		Units × 10 ^{3}/mg protein
Stem (from microcultures)	58.21		18.0
Leaf (from microcultures)	42.98		5.4
Stem Callus (colored)	4.69		4.27
Stem Callus (colorless)	0.813		1.56
Leaf Callus (colored)	4.60		3.96
Leaf Callus (colorless)	1.89	1.39	

oratory have indicated that higher sucrose levels were necessary to induce anthocyanin production in cranberry cultures (Madhavi et al., 1993). Total anthocyanin levels increased from 0–3.0% on increasing sucrose levels from 2–5% in the production medium. Anthocyanin profiles were simpler, similar to other *in vitro* systems like rabbiteye blueberry (Nawa et al., 1993) and grapes (Yamakawa et al., 1983). The decline in anthocyanin concentration after 12 days could be mainly due to depletion of nutrients in the culture medium and has been reported in rabbiteye blueberry cell cultures (Nawa et al., 1993). It was interesting to note that fruit callus had low levels of anthocyanins and a similar anthocyanin profile to callus cultures derived from vegetative explants, indicating that fruit callus was physiologically distinct from differentiated fruit tissue.

Qualitative and quantitative differences in anthocyanin accumulation in fruits *in vivo* and callus cultures *in vitro* could be due to the physiological nature of explant tissues used for callus induction. In cranberry fruits, anthocyanins are localized in the epidermis and the fruit callus culture was derived from mesocarp tissue, devoid of anthocyanins. Vegetative tissues both in the greenhouse and microculture had traces of anthocyanins and qualitatively only cyanidin glycosides. Peonidins differ from cyanidins by a single methyl group substitution at the 3' position in the B ring and are generally considered to be derived from cyanidins by a methylation reaction. Methylation reactions in the flavonoid biosynthetic pathway are catalysed by specific *O*-methyltransferases with S-adenosyl-L-methionine as the methyl donor (Poulton, 1981). *O*-methyltransferases specific for glucosylated and acylated anthocyanidins have been demonstrated in *Petunia hybrida* (Jonsson et al., 1982).

No information is available on properties of methyltransferases in cranberries. Our results, however, indicated that the callus cultures and vegetative tissues may lack methyltransferases specific for anthocyanins or the methyl donor. In general, the metabolic constraints physiological regulations operative in the callus cultures seemed to be similar to the source explants. Our results also indicated that some constraints could be overcome by manipulation of cultural conditions. Anthocyanin accumulation was lower *in vitro* as compared to the fruit extract but was clearly higher than explant tissues (Table 1). Establishment of suspension cultures and use of selection protocols may help in developing intensely pigmented cell lines as with *Vitis hybrida* (Yamakawa et al., 1983), *Ajuga reptans* (Callebaut et al., 1988), and *Daucus carota* (Kinnersley and Dougall, 1980).

The ethyl acetate-extractable fraction was analysed mainly to get an indication of the presence of flavonoids other than anthocyanins. The chromatograms were monitored at 280 nm since all compounds absorbed at that wavelength and the area percentage was also expressed in the same wavelength (Table 2). This slightly underestimates flavonols and their derivatives which absorb more strongly at 350–370 nm. However, this method of representation based on spectral properties gave a good indication of differences *in vivo* and *in vitro*. Cranberry fruits are a rich source of flavonols (Bilyk and Sapers, 1986) and our results indicated a higher percentage of flavonols. Callus cultures from vegetative explants were similar to the explants and had a higher percentage of cinnamic acids and catechins. Fruit callus differed from fruit in that it had a lower percentage of flavonols. Callus cultures accumulated flavonols only on exposure to light (Fig. 5).

Proanthocyanidins are condensation products of monomeric leucoanthocyanidins and catechins and include dimers, oligomers, and polymers. Those with molecular weights >7000 become insoluble in organic and aqueous solvents (Czochanska et al., 1979; Haslam, 1980). Proanthocyanidins extractable in cold organic solvents have been reported in cranberry fruits (Wang et al., 1978; Foo and Porter, 1981). We have demonstrated the presence of insoluble proanthocyanidins both *in vivo* and *in vitro* indirectly by hydrolysis in HCl methanol. *In vivo*, fruits seemed to have a low level of insoluble proanthocyanidins which could be mainly related to the degree of fruit ripening (Haslam, 1977). *In vitro*, callus cultures from vegetative explants showed lower accumulation of insoluble proanthocyanidins as compared to explants. Fruit callus again differed from fruit and had a higher level of insoluble proanthocyanidins and was similar to cultures from vegetative explants. Light was not a limiting factor for proanthocyanidin accumulation in our cultures (Table 1). Light independent accumulation of high molecular weight proanthocyanidins has been reported in other cell culture systems like *Cryptomeria japonica* (Ishikura and Teramoto, 1983), *Fagopyrum esculentum* (Moumou et al., 1992) and *Vaccinium ashei* (Nawa et al., 1993). Cultural conditions also reportedly influence

the accumulation of proanthocyanidins in cell cultures (Zaprometov, 1988). Proanthocyanidins and anthocyanins share common intermediates in the biosynthetic pathway. Hence, inhibition of proanthocyanidin synthesis by manipulating cultural conditions may enhance anthocyanin accumulation.

Phenylalanine ammonia-lyase catalyzes the formation of *trans*-cinnamic acid, the first phenylpropanoid, by the deamination of L-phenylalanine and provides the link between primary metabolism and the phenylpropanoid pathways (Ebel and Hahlbrock, 1982). The enzyme may also have a regulatory effect in anthocyanin accumulation (Hahlbrock, 1981). Sapers et al. (1987) demonstrated PAL activity in the skin of cranberry fruits and reported that the activity did not correlate with anthocyanin accumulation. Our studies indicated that the observation could be extended to vegetative parts of the plant. In vitro, dark grown cultures had a basal level of activity which was enhanced by illumination (Table 3). Exposure to light induced the formation of anthocyanins and flavonols. PAL and other enzymes in the flavonoid biosynthetic pathway are highly light inducible (McClure, 1975) and our results confirmed this. However, cultures also accumulated proanthocyanidins in the absence of light, which implied the synthesis of dihydroflavonols and leucoanthocyanidins, common intermediates for anthocyanins, flavonols, and proanthocyanidins in the biosynthetic pathway. This indicated that the increase in PAL activity could be responsible for an enhanced precursor pool in the biosynthetic pathway.

REFERENCES

Bilyk, A. and Sapers, G.M. 1986. Varietal differences in the quercetin, kaempferol, and myricetin contents of highbush blueberry, cranberry, and thornless blackberry fruits. J. Agric. Food Chem. 34: 585–588.

Bradford, M.M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochemistry 72: 248–254.

Callebaut, A., Hendrickx, G., Voets, A.M., and Perwez, C. 1988. Production of anthocyanins by cell cultures of *Ajuga reptans*. Med. Fac. Landbouww Rijksuniv Gent. 53: 1713–1715.

Callebaut, A., Hendrickx, G., Voets, A.M., and Motte, J.C. 1990. Anthocyanins in cell cultures of *Ajuga reptans*. Phytochemistry 29: 2153–2158.

Cormier, F. and Do, C.B. 1993. *Vitis vinifera L. (Grapevine): In Vitro Production of Anthocyanins, Biotechnology in Agriculture and Forestry*, Vol. 24, Y.P.S. Bajaj (Ed.), p. 373–386. Springer-Verlag, Berlin Heidelberg.

Crouch, N.R., Van Staden, L.F., Drewes, F.E., and Meyer, H.J. 1993. Accumulation of cyanidin 3-glucoside in callus and cell cultures of *Oxalis reclinata*. J. Plant Physiol. 142: 109–111.

Czochanska, Z., Foo, L.Y., Newman, R.H., Porter, L.J., Thomas, W.A., and Jones, W.T. 1979. Direct proof of a homogeneous polyflavan-3-ol structure for polymeric proanthocyanidins. J. Chem. Soc. Chem. Commun. 375–377.

Do, C.B. and Cormier, F. 1991. Effects of low nitrate and high sugar concentrations on anthocyanin content and composition in grape (*Vitis vinifera* L.) cell suspension. Plant Cell Reports 9: 500–504.

Ebel, J. and Hahlbrock, K. 1982. Biosynthesis. In *The Flavonoids: Advances in Research*, J.B. Harborne and T.J. Mabry (Ed.), p. 641–679. Chapman and Hall, New York.

Foo, L.Y. and Porter, L.J. 1981. The structure of tannins of some edible fruits. J. Sci. Food Agric. 32: 711–716.

Francis, F.J. 1982. Analysis of anthocyanins. In *Anthocyanins as Food Colors*, P. Markakis (Ed.), p. 181–207. Academic Press, New York.

Fuleki, T. and Francis, F.J. 1967. The co-occurrence of monoglucosides and monogalactosides of cyanidin and peonidin in the American cranberry, *Vaccinium macrocarpon*. Phytochemistry 6: 1705–1708.

Gamborg, O.L., Miller, R.A., and Ojima, K. 1968. Nutrient requirements of suspension cultures of soybean root cells. Exp. Cell Res. 50: 151–158.

Hahlbrock, K. 1981. Flavonoids. In *The Biochemistry of Plants, Vol. 7, Secondary Plant Products*, P.K. Stumpf and E.E. Conn (Ed.), p. 425–456. Academic Press, New York.

Haslam, E. 1977. Symmetry and promiscuity in procyanidin biochemistry. Phytochemistry 16: 1625–1640.

Haslam, E. 1980. *In vino veritas*: Oligomeric procyanidins and the aging of red wines. Phytochemistry 19: 2577–2582.

Havir, E.A. and Hanson, K.R. 1971. L-Phenylalanine ammonia-lyase (potato tubers), Methods in Enzymology 17a: 575–578.

Hirasuna, T.J., Shuler, M.L., Lackney, V.K., and Spanswick, R.M. 1991. Enhanced anthocyanin production in grape cell cultures. Plant Science 78: 107–120.

Hong, V. and Wrolstad, R.E. 1990. Use of HPLC separation/photodiode array detection for characterization of anthocyanins. J. Agric. Food Chem. 38: 708–715.

Hong, Y.C., Read, P.E., Harlander, S.K., and Labuza, T.P. 1989. Development of a tissue culture system from immature strawberry fruits. J. Food Sci. 54: 388–392.

Ilker, R. 1987. *In vitro* pigment production: an alternative to color synthesis. Food Technol 41(4): 70–72.

Ishikura, N. and Teramoto, S. (1983). Procyanidins and catechin from callus and cell suspension cultures of *Cryptomeria japonica*. Agric. Biol. Chem. 47: 421–423.

Jonsson, L.M.V., Aarsman, M.E.G., Schram, A.W., and Bennink, G.J.H. 1982. Methylation of anthocyanins by cell-free extracts of flower buds of *Petunia hybrida*. Phytochemistry 21: 2457–2459.

Kinnersely, A.M. and Dougall, D.K. 1980. Increase in anthocyanin yield from wild-carrot cell cultures by a selection system based on cell-aggregate size. Planta 149: 200–204.

Lloyd, G. and McCown, B.H. 1981. Commercially-feasible micropropagation of mountain laurel, *Kalmia latifolia* by use of shoot tip culture. Proc. Int. Plant Prop. Soc. 30: 421–427.

Madhavi, D.L., Smith, M.A.L., and M.D. Berber-Jimenez, M.D. 1993. Anthocyanins from cell cultures of cranberry. Proceedings of the First International Symposium on Natural Food Colors, Univ. Massachusetts, Amherst, MA, 283–286.

Markham, K.R. 1982. *Techniques of Flavonoid Identification*. Academic Press, New York.

Marcotrigiano, M. and McGlew, S.P. 1991. A two-stage micropropagation system for cranberries. J. Amer. Soc. Hort. Sci. 116: 911–916.

McClure, J.W. 1975. Physiology and Functions of Flavonoids. In *The Flavonoids*, J.B. Harborne, T. Mabry, and H. Mabry (Ed.), p. 970–1055. Chapman and Hall, London.

Mori, T., Sakurai, M., Shigeta, J., Yoshida, K., and Kondo, T. 1993. Formation of anthocyanins from cells cultured from different parts of strawberry plants. J. Food Sci. 58: 788–792.

Moumou, Y., Trotin, F., Vasseur, J., Vermerrsch, G., Guyon, R., Dubois, J., and Pinkas, M. 1992. Procyanidin production by *Fagopyrum esculentum* callus culture. Planta Med. 58: 516–519.

Nawa, Y., Asano, S., Motoori, S., and Ohtani, T. 1993. Production of anthocyanins, carotenoids, and proanthocyanidins by cultured cells of rabbiteye blueberry (*Vaccinium ashei* Reade). Biosci. Biotech. Biochem. 57: 770–774.

Nozue, M., Kawai, J., and Yoshitama, K. 1987. Selection of a high anthocyanin-producing cell line of sweet potato cell cultures and identification of pigments. J. Plant Physiol. 129: 81–88.

Ozeki, Y. and Komamine, A. 1985. Effects of inoculum density, zeatin, and sucrose on anthocyanin accumulation in a carrot suspension culture. Plant Cell Tissue Org. Cult. 5: 45–53.

Poulton, E.J. (1981). Transmethylation and demethylation reactions in the metabolism of secondary plant products. In *The Biochemistry of Plants, Vol. 7, Secondary Plant Products*, P.K. Stumpf and E.E. Conn (Ed.), p. 667–723. Academic Press, New York.

Sapers, G.M., Matulaitis, R.M., and Beck, J.A. (1987). Detection of phenylalanine ammonia-lyase activity in the skin of blueberry and cranberry fruits. J. Food Sci. 52: 155–158.

Serres, R., Stang, D., McCabe, D., Russel, D., Mahr, D., and McCown, B.H. 1992. Gene transfer using electric discharge particle bombardment and recovery of transformed cranberry plants. J. Amer. Soc. Hort. Sci. 117: 174–180.

Shuler, M.T., Hirasuna, T., Prince, C., and Bringi, V. 1990. Bioreactor considerations for producing flavors and pigments from plant tissue culture. In *Biotechnology and Food Process Engineering*, H. Schwartzberg and M. Rao (Ed.), p. 45–65. Marcel Dekker, Inc., New York.

Stafford, A. 1991. The manufacture of food ingredients using plant cell and tissue cultures. Trends in Food Sci. Tech. 2: 116–122.

Wang, P.L., Du, C.T., and Francis, F.J. 1978. Isolation and characterization of polyphenolic compounds in cranberries. J. Food Sci. 43: 1402–1404.

Wilson, G. 1990. Screening and selection of cultured plant cells for increased yields of secondary metabolites. In *Plant Cell Line Selection*, P.J. Dixon (Ed.), p. 187–213. VCH, Weinheim.

Yamakawa, T., Ishida, K., Kato, S., Kodama, T., and Minoda, Y. 1983. Formation and identification of anthocyanins in culture cells of *Vitis* sp. Agric. Biol. Chem. 47: 997–1001.

Zaprometov, M.N. 1988. Proanthocyanidins and catechins. Ch. 4, in *Cell Culture and Somatic Cell Genetics of Plants*, Vol. 5, F. Constabel and I.K. Vasil (Ed.), p. 77–88.

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Combined Stress Effects on Growth of *Zygosaccharomyces rouxii* from an Intermediate Moisture Papaya Product

M.S. TAPIA DE DAZA, C.E. AGUILAR, V. ROA, and R.V. DÍAZ DE TABLANTE

ABSTRACT

Stress factors studied included 0.95 a_w adjusted with sucrose and glycerol, pH (4.0, 3.5, and 3.0), potassium sorbate (0, 500, 1000, and 1500 ppm) and sodium bisulfite (0, 100, and 150 ppm). These were tested on a strain of *Zygosaccharomyces rouxii* isolated from an intermediate moisture (IM) papaya product. *Z. rouxii* was very sensitive to SO_2. A combination of 100 ppm SO_2, 500 ppm sorbate and pH 4.0 is recommended to prevent spoilage of IM fruits by *Z. rouxii*. The minimum a_w for growth in sucrose-glycerol broth was between 0.62 and 0.64 in the absence of antimicrobials and in the range of optimal pH for this yeast.

Key Words: *Zygosaccharomyces*, yeast, papaya, intermediate moisture, water activity

INTRODUCTION

OSMOTOLERANT YEASTS can spoil foods with low to intermediate water activity, high acidity, low redox potential, high carbon/nitrogen ratio foods, and do not compete much with other microorganisms in nonselective environments (Restaino et al., 1983). Thus, such yeasts are important in spoilage of foods where such stress factors are used to control microbial growth, as with intermediate moisture products. This applies also to shelf-stable high moisture fruit products. Such processing techniques (Levy et al., 1985; García et al., 1985; Jayaraman, 1987; CYTED-D, 1990; Alzamora et al., 1988, 1993) are being applied especially in developing countries where these types of fruit products have potential markets and contribute to reduction of post-harvest losses and diversification of local fruit industries. To optimize such preservation processes, information is needed about the combined effect of stress factors on growth of microorganisms that are important in potential spoilage of finished fruit products (Cerruti et al., 1990). *Zygosaccharomyces rouxii* [syn: *Saccharomyces rouxii* (Yarrow, 1984)] is the most common spoilage organism of osmotolerant yeasts, and thus an organism of choice for microbial challenge studies of shelf stable fruit products.

Our objective was to investigate the response of a strain of *Z. rouxii* (isolated from an intermediate moisture, 0.95 a_w papaya product in laboratory media to stress factors such as water activity, acidity and chemical preservatives. The minimum a_w for growth in sucrose-glycerol broth was also investigated since glycerol and sucrose are widely used humectants in IMF technology. Tolerance of yeasts to low a_w depends on the solute used for a_w control.

MATERIALS & METHODS

Organism

Z. rouxii (strain TA) from the culture collection of the Instituto de Ciencia y Tecnología de Alimentos, Universidad Central de Venezuela, Caracas, was used. The strain had been isolated originally from an intermediate moisture papaya product containing 0.1% potassium sorbate and identified in the Departamento de Micología of the Instituto Nacional de Higiene Rafael Rangel, Caracas. The stock culture was maintained in slants of yeast extract sucrose (YES) 50 agar; pH 4.5 (adjusted with phosphoric acid); 50% (w/w) commercial sucrose; 0.5% yeast extract; 1.5% (w/w) agar. This is a modification of the yeast extract glucose (YEG) medium described by Jermini and Schmidt-Lorenz (1987).

Inocula

The inocula for growth studies were prepared by transferring a loopful of the stock culture to 100 mL of yeast extract sucrose (YES) 50 broth in 250 mL Erlenmeyer flasks on a rotary shaker at 28°C. Inoculation suspensions were prepared from 48-h cultures, diluted with peptone water plus 20% (w/w) glycerol to 10^5 cells/mL. Concentration of suspensions was checked with a Neubauer counting chamber. Counts in chamber were corroborated by plating on YES 50 agar plates. Sterile experimental media (100g) for growth studies were inoculated with 0.1 mL of these suspensions.

Media for minimal water activity studies

These studies were conducted in glycerol-sucrose broth (pH 4.0). The basal medium consisted of 19% (w/w) water, 47.6% glycerol, 28.6% sucrose and 4.8% yeast extract and peptone to give a_w-0.60. The wt of water needed to gradually increase water activity to 0.75 was calculated from the working adsorption isotherm (0.60–0.75 a_w, 25°C) of the basal medium (Fig. 1) obtained as described by Labuza (1988). Water activity was determined with a psycrometric instrument manufactured by Decagon Devices Inc. (Pullman WA.) Model CX-1, calibrated as described by Roa and Tapia (1990), and moisture content by gravimetric determination in vacuum oven at 70°C. Test media with the following water activities were prepared: 0.60, 0.62, 0.64, 0.66, and 0.68. Equations for fitting water sorption isotherms were used (Boquet et al., 1978). Linear regression curves were determined from experimental data to select the mathematical model to give best correlation factors.

Two additional test media were prepared to give water activities of 0.85 and 0.95, using a computer program in Basic language. The program was designed in our laboratory, to calculate final water activity values based upon given solutes and water concentrations. The program estimates the contribution of each solute to water activity depression through Norrish's (1966) and Ross's (1975) equations for single nonelectrolyte and multicomponent solutions respectively. Predicted water activities of media were instrumentally checked and good agreement was always found between predicted and experimental values.

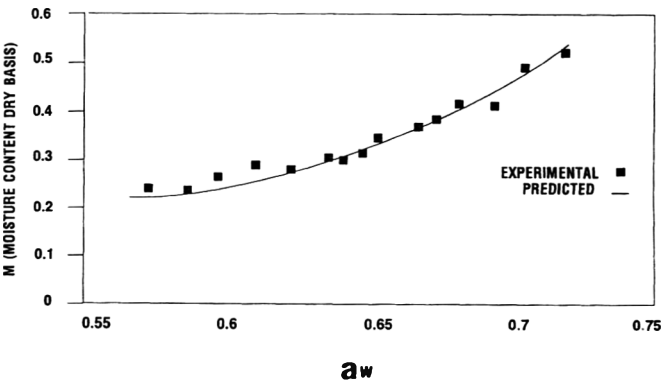

Fig. 1—Adsorption isotherm for sucrose:glycerol broth basal medium at 28°C. (■) experimental values; line-predicted values.

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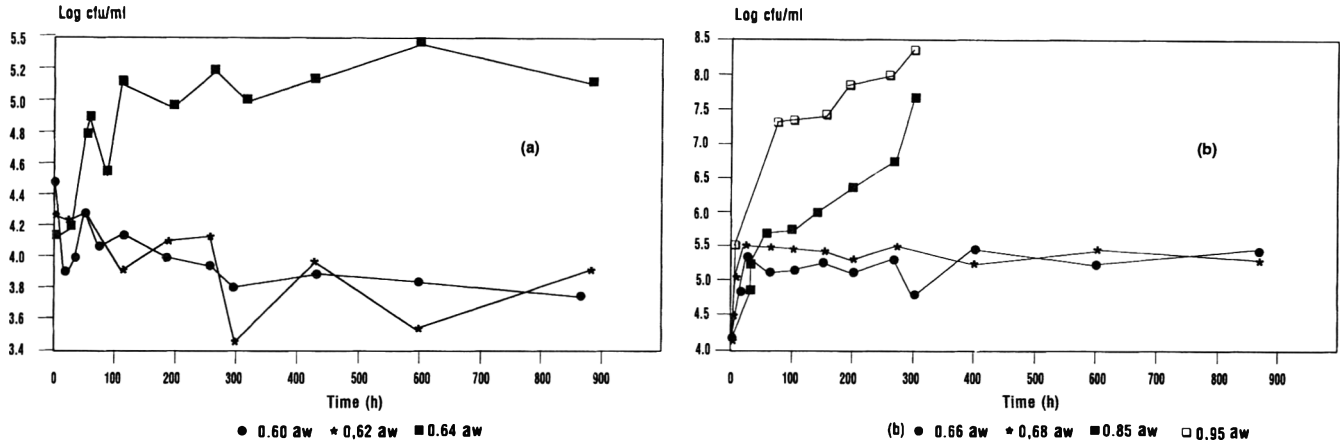

Fig. 2—Effect of water activity on growth of *Z. rouxii* in sucrose:glycerol broth (pH 4.0) during 840 hr incubation at 28°C. (Left) ● 0.60 a_w * 0,62 a_w ■ 0.64 a_w; (Right) ● 0.66 a_w * 0,68 a_w ■ 0.85 a_w; □ 0.95 a_w

Media for response to stress factors

The test medium sucrose:glycerol (0.95a_w) prepared as described, was used for studies of response to stress factors. Phosphoric acid was added to adjust pH to 4.0, 3.5, and 3.0. The pH was checked with a glass electrode on a Extech Model 671 pH meter (Boston, MA., U.S.A.). Sodium metabisulfite (Merck, Darmstadt, West Germany) and potassium sorbate (food grade) were added to the desired final concentrations of antimicrobials (100 and 150 ppm SO_2 and 500, 1000, and 1500 ppm potassium sorbate). Blanks with no antimicrobials added were also prepared. All media were sterilized by filtration (pore size 0.2 μm).

Medium for enumeration

YES 50 agar was used as plating medium to enumerate viable yeast cells by pour plating. At time intervals (0, 3, 6, 9, 12, 20, 40, 60, 80, 120, 180, 240, 300, 420, 600, and 840 hr for studies of minimal a_w for growth and for estimation of generation times; and 0, 3, 6, 12, 24, 48, 216, and 360 hours for studies of response to stress factors) 1.0 mL aliquots were taken from the inoculated test media. These were serially diluted with peptone water plus 20% (w/w) glycerol to avoid osmotic shock, and plated in duplicate. Plates were incubated at 28°C for five days. Counts were transformed to $\log_{10}$ colony forming units (CFU/mL).

Statistical analysis

Data are means of values from three replicate experiments performed in duplicate on studies of minimal a_w for growth and on response to stress factors. Analysis of variance used Statgraphics (Version 6, Statistical Graphics Corporation). Mean counts on YES 50 agar were compared ($P \leq 0.05$) using Least significant differences (LSD). Linear regression curves were determined from data when exponentially growing *Z. rouxii* cells were obtained and generation times (hr) were computed from those curves.

Residual sorbic acid and SO_2

Residual sorbic acid and sulfur dioxide were determined in test media used in response studies to stress factors. Sorbate was determined using the colorimetric method described by Nury and Bolin (1963) and sulfur dioxide by the modified Monier-Williams method (**AOAC**, 1980).

Table 1—Generation times of *Z. rouxii* in sucrose-glycerol broth (pH 4.0) during 60 hr incubation at 28°C

	a_w				
	0.64	0.66	0.68	0.85	0.95
Generation time (hr)[a]	17.42	16.79	16.20	7.21	6.20

[a] Generation times represent mean values from three replicate experiments performed in duplicate.

RESULTS & DISCUSSION

Minimal a_w for growth

The effect of water activity on growth of *Z. rouxii* has been reported. The minimum a_w range in a fructose syrup at pH 4.8 and in glucose (von Schelhorn, 1950; Tilbury, 1976) broths was 0.62 to 0.65, whereas in NaCl, a minimal value for growth of 0.86 has been reported (Onishi, 1963). The type solute used to reduce a_w of growth media influenced both amount and rate of fungal growth (Anand and Brown, 1968; Warth, 1986) as well as growth response to antimicrobials (Bills et al., 1982, Lenovitch et al., 1988. The a_w adjusted with a mixture sucrose-glycerol (3:5) affected the growth of *Z. rouxii* at pH 4.0 and 28°C Fig. 2. The yeast was inhibited at a_w 0.60 and 0.62 while at a_w 0.64, 0.66, and 0.68 slow growth was observed. At a_w 0.85 and 0.95 the yeast grew more rapidly, reaching 10^7 CFU/mL, though at different rates. Tilbury (1980) reported minimum a_w 0.65 for growth of *Z. rouxii* in sucrose/glycerol syrups incubated 12 wk at 27°C. Information on solutes ratios used was not indicated.

Tilbury (1980) also reported differences in growth rates depending on water activity of the syrups. He considered the a_w at which final *Z. rouxii* counts were 1.5–2.0 times greater than original counts (after 12 wk @ 27°C), as the minimal value for growth. If values were <1.5 the original count, as with 0.60 and 0.625 a_w, it was considered as no growth. At higher water activities (0.75 and 0.80 a_w), final counts were >20 times greater than the original count. Our results indicated a minimal value for growth between 0.62 and 0.64. Generation times were calculated (Table 1) for *Z. rouxii* in sucrose-glycerol media at water activities above the minimum for growth. Generation times from various published sources (Table 2) for *Z. rouxii* were compared. Generation times we observed at high a_w were somewhat higher than reported values. This could be attributed to the combined effects of both solutes.

Z. rouxii exhibits low growth rates and extended lag phases at low water activity. Horner and Anagnostopoulos (1973) measured radial growth rates and lag phases of colonies of *Z. rouxii* on sucrose agar over a_w 0.997 to 0.90. The lag phase for

Table 2—Mean generation times reported for *Z. rouxii* in laboratory media with a_w adjusted with different solutes

Solute	a_w or solute conc	Mean generation time	References
Glucose	0.935	2.15 h	Tilbury (1976, 1980)
Glucose	30%	4.5 h	Moran and Witter (1979)
Fructose	0.62	two mo	von Schelhorn (1950)
Glucose and	0.96	2.55 h	Restaino et al. (1983)
sucrose	0.97	2.35 h	
Sucrose	0.98	1.30 h	Tilbury (1976, 1980)

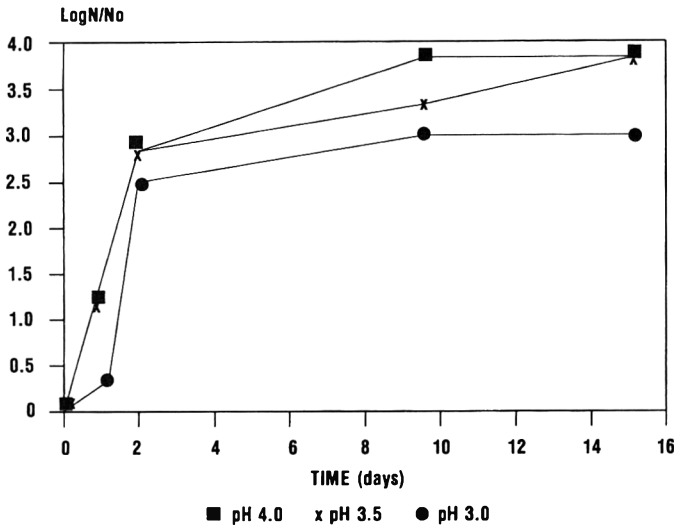

Fig. 3—Effect of pH (adjusted with phosphoric acid) on growth of *Z. rouxii* in sucrose:glycerol broth (0.95 a_w) during 15 days incubation at 28°C. ■ pH 4.0 x pH 3.5 ● pH 3.0

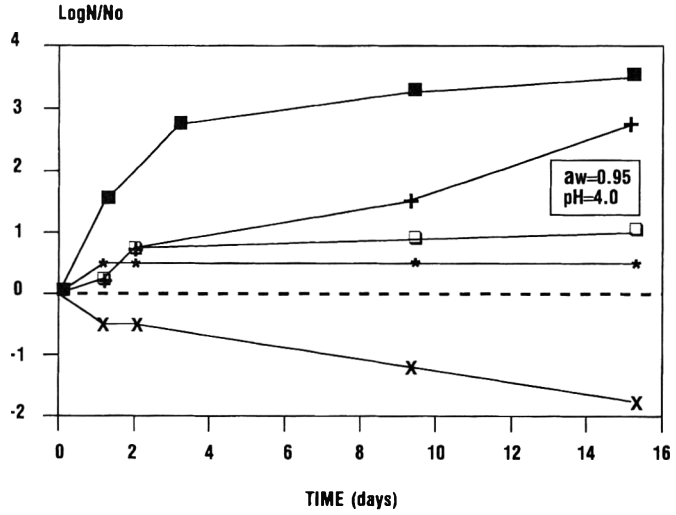

Fig. 4—Effect of combined stress factors (potassium sorbate and sulfur dioxide) on growth of *Z. rouxii* in sucrose:glycerol broth. (■) 0 ppm sorbate; (+) 500 ppm sorbate; (□) 1000 ppm sorbate; (*) 0 ppm sorbate and 100 ppm SO_2; (X) 500 ppm sorbate and 100 ppm SO_2.

colony formation doubled to 40 hr at a_w 0.90 compared with 20 hr at a_w 0.960. They also studied the effect of a_w controlled by sucrose and glycerol separately on growth rates and lag phases of three yeasts including *Z. rouxii*. This yeast exhibited the lowest growth rate, the longest lag (*ca.* 20 hr) over the high a_w range, and the lowest rate of increase in lag in sucrose. Glycerol was a little less inhibitory than sucrose. However, when mixtures of solutes were used (glycerol and sucrose) results were almost identical to those predicted from single solute experiments. Golden and Beuchat (1990) recommended that glucose be used to reduce a_ws of media during enumeration of *Z. rouxii*, since it is less inhibitory to this yeast than glycerol, sucrose and sorbitol. Abdul-Raouf et al. (1994) reported that glucose diluents resulted in better recovery of *Z. rouxii* from IM foods than did glycerol diluents.

Response to stress factors

It is possible to inhibit development of microorganisms at a_w levels higher than the minimal a_w for growth by combinations of microbial stress factors (Cerruti et al., 1990). Effective synergistic effects may occur when some preservation factors are used in combinations. Any restriction of energy supply will tend to be especially synergistic when lowered water activity is used for preservation (Gould et al. 1983). Care must be taken, not to misidentify additive interactions as synergistic since some antimicrobials interactions can be antagonistic (Parish and Davidson, 1993). Among some food-related antimicrobial combinations reported as antagonistic in broth media are SO_2 and benzoate and SO_2 and sorbate (Parish and Carroll, 1988).

Z. rouxii was grown in laboratory media containing sucrose and glycerol (0.95a_w) to study its response to stress factors. The effect of pH (4.0 to 3.5 and 3.0), amount of potassium sorbate (0, 500, 1000 and 1500 ppm) and sodium bisulfite (0, 100 and 150 ppm) were studied. Yeast counts on YES 50 agar were reported as log N/N_0, where N is the number of yeasts (cfu/mL) at a specific time and N_0 is the number of yeasts cfu/mL at time zero.

Growth response of *Z. rouxii* was compared (Fig. 3) for three pH values at 0.95 a_w, in the absence of sulfite and sorbate. English (1954) reported a high tolerance of this yeast to a wide range of acidity. Restaino et al. (1983) found an optimum pH range of 3.5 to 5.5, much wider than that reported by Tilbury. In our study, a pH of 3.0 had a more inhibitory effect indicated by significantly ($P \leq 0.05$) smaller counts of the yeast at that pH, than those at pH 4.0 or 3.5.

Effects of potassium sorbate (SK) and sulfur dioxide on growth of *Z. rouxii* in laboratory media (0.95a_w adjusted with sucrose) were compared (Fig. 4). Addition of 500 ppm SK retarded growth especially during the first hours of incubation. At the end of the incubation period in the presence of 500 ppm SK, counts grew higher ($P \leq 0.05$) approaching those obtained in absence of sorbate. This suggests possible adaptation to sorbate. Residual sorbate levels were calculated in the test media. The initial undissociated fraction (that reportedly with highest antimicrobial activity even when activity was also atributed to dissociated molecules to a lesser extent; Booth and Kroll, 1989) was expected to have an important activity because the pH of the test medium was 4.0. About 93% of the 500 ppm of sorbic acid initial concentration was detected indicating nearly all of it remained in the test medium. Increasing sorbate to 1000 ppm resulted in evident growth inhibition (Fig. 4) while with 1500 ppm in the test medium, no growth occurred (data not shown in figure).

Addition of 100 ppm sulfur dioxide (in absence of sorbate) had a marked effect on growth. Significantly ($P \leq 0.05$) retarded growth occurred compared with growth in the previous test media (Fig. 4). This effect of sulfite at pH 4.0 was more pronounced with decreasing pH of 3.5 and 3.0 (data not presented). Residual SO_2 levels were determined (15 days) in the test media with 20 and 30% of the original sulfite concentration detected. Cerruti et al. (1988), reported that the lethal effect of 100 ppm SO_2 on a strain of *S. cerevisiae* when added to the heating medium was clearly greater than that of 100 ppm potassium sorbate. They also found that a mixture of 50 ppm sorbate and 50 ppm SO_2 acted synergistically with heat to rapidly inactivate *S. cerevisiae* at 50 or 55°C. In our study, when 500 ppm potassium sorbate was added along with 100 ppm SO_2 an inhibitory ($P \leq 0.05$) effect was obtained at pH 4.0 as compared to counts of yeast in presence of each antimicrobial alone. This suggested a synergistic (not antagonistic as reported by Parish and Carroll, 1988) interaction of these antimicrobials. Addition of 150 ppm SO_2 resulted in total inhibition of the yeast (not shown in figure). Sajur (1985) reported growth of *S. cerevisiae* in peach homogenates in the presence of 1000 ppm SK and no growth when 150 ppm SO_2 was added.

Cerruti et al. (1990) studied the effect of reduced water activity, pH, and potassium sorbate on growth of *S. cerevisiae* in model systems representing preservation effects by combined methods. They reported different combinations of a_w, pH and potassium sorbate led to inhibition of the organism in laboratory

media. In our case we did not vary a_w from 0.95 since we were developing an intermediate moisture fruit product in that water activity range. A combination of 100 ppm SO_2, 500 ppm sorbate and pH 4.0 based on our results, is recommended to prevent spoilage of intermediate moisture fruit products by *Z. rouxii*. Studies are also being conducted in model fruit systems instead of laboratory model media.

REFERENCES

Abdul-Raouf, U.M., Hwang, C.A., and Beuchat, L.R. 1994. Comparison of combinations of diluents and media for enumerating Zygosaccharomyces rouxii in intermediate water activity foods. Lett. Appl. Microbiol. 9: 28–31.

Alzamora, S.M., Gerchenson, L.N., Cerruti, P., and Rojas, A.M. 1989. Shelf-stable pineapple for long term non refrigerated storage. Lebensm. Wiss. Technol. 22: 233.

Alzamora, S.M., Tapia, M.S., Argaíz, A. and Welti. J. 1993. Application of combined methods technology in minimally processed fruits. Food Res. Intntl. 26: 125.

Anand, J.C. and Brown, A.D. 1968. Growth rate patterns of the so called osmophilic and non-osmophilic yeasts in solutions of poliethylene glycol. J. Gen. Microbiol. 52: 205.

AOAC. 1980. *Official Methods of Analysis*, 11th ed. Association of Official Agricultural Chemists, Washington, DC.

Bills, S., Restaino, L., and Lenovitch, L.M. 1982. Growth response of an osmotolerant sorbate-resistant yeast, *Saccharomyces rouxii*, at different sucrose and sorbate levels. J. Food Prot. 45: 1120.

Booth, I.R. and Kroll, R.G. 1989. The preservation of foods by low pH. Ch. 6. In *Mechanisms of Action of Food Preservation Procedures*, G.W. Gould (Ed.), p. 141. Elsevier Applied Science, London-New York.

Boquet, R., Chirife, J., and Iglesias, H. 1978. Equations for fitting water sorption isotherms of foods. II. Evaluation of various two parameters models. J. Food Sci. 13: 319.

Cerrutti, P., Alzamora, S.M., and Chirife, J. 1988. Effect of potassium sorbate and sodium bisulfite on thermal inactivation of *Saccharomyces cerevisiae* in media of lowered water activity. J. Food Sci. 53: 1911.

Cerruti, P., Alzamora, S., and Chirife, J. 1990. A multiparameter approach to control the growth of *Saccharomyces cerevisiae* in laboratory media. J. Food Sci. 55: 839.

CYTED-D Program. 1991. Desarrollo de alimentos de humedad intermedia importantes para Iberoamérica. Sub-Proyecto Frutas y Hortalizas. Universidad de las Américas, Puebla, México.

English, M.P. 1954. Some observations on the physiology of *Saccharomyces rouxii* Boutroux. J. Gen. Microbiol. 10: 328.

García, R., de Arriola, M.C., de Porres, E., and Rolz, C. 1985. Process for banana puree preservation at rural level. Lebensm.-Wiss. u Technol. 18: 323.

Gould, G.W., Brown, M.H., and Fletcher, B.C. 1983. Mechanisms of action of food preservation procedures. In *Food Microbiology. Advances and Prospects*, T.A. Roberts and F.A. Skinner (Ed.), p. 67. Academis Press, New York.

Golden, D.A., and Beuchat, L.R. 1990. Colony formation by sublethally heat injured *Zigosaccharomyces rouxii* as affected by solutes in the recovery medium and procedure for sterilizing medium. Appl. Environ. Microbiol. 2319–2326.

Horner, K.J. and Anagnostopoulos, G.D. 1973. Combined effects of water activity, pH, and temperarture on the growth and spoilage potential of fungi. J. Appl. Bacteriol. 36: 427.

Jayaraman, K.S. 1988. Development of intermediate moisture tropical fruit and vegetable products—Technological problems and prospects. In *Food Preservation by Moisture Control*, C.C. Seow (Ed.), p. 175. Elsevier Applied Science, London, New York.

Jermini, M.F.G. and Schmidt-Lorenz, W. 1987. Cardinal temperatures for growth of osmotolerant yeasts in broths at different water activities values. J. Food Prot. 50: 473.

Labuza, T. 1988. Moisture sorption: Practical aspects of isotherm measurement and use. *Operator's manual CX-1 Water Activity System*, Decagon Devices, Inc., Pullman, WA.

Lenovitch, L.M., Buchanan, R.L., Worley, N.J., and Restaino, L. 1988. Effect of solute type on sorbate resistance in *Zygosaccharomyces rouxii*. J. Food Sci. 53: 914.

Levy, A., Gagel, S., and Juven, B.J. 1985. Intermerdiate moisture tropical fruit products for developing countries. II. Quality characteristics of papaya. J. Food Technol. 30: 163.

Moran, J.W. and Witter, L.D. 1979. Effect of sugars on D-arabitol production and glucose metabolism in *Saccharomyces rouxii*. J. Bacteriol. 138: 823.

Norrish, R.S. 1966. An equation for the activity coefficients and equilibrium relative humidities of water in confectionary syrups. J. Food Technol. 1: 25.

Nury, F.S. and Bolin, H.R. 1963. Colorimetric assay for potassium sorbate in dried fruits. J. Food Sci. 27: 370.

Onishi, H. 1963. Osmophilic yeasts. Adv. Food Res. 12: 53.

Parish, M.E. and Carroll, D.E. 1988. Effects of combined antimicrobials agents on fermentation initiation by *Saccharomyces cerevisiae* in a model broth system. J. Food Sci. 53: 240.

Parish, M.E. and Davidson, M.E. 1993. Methods for evaluation. Ch. 17. In *Antimicrobials in foods*, P.M. Davidson and A.L. Branen (Ed.), p. 608. Marcel Dekker, Inc., New York.

Restaino, L., Bills, S., Tscherneff, and Lenovitch, L. 1983. Growth characteristics of *Saccharomyces rouxii* isolated from chocolate syrup. 45: 1614.

Roa, V. and Tapia de Daza, M.S. 1991. Evaluation of water activity measurements with a dew point electronic humidity meter. Lebensm. Wiss. Technol. 24: 208.

Ross, K.D. 1975. Estimation of water activity in intermediate moisture foods. Food Technol. 29: 26.

Sajur, S. 1985. Preconservación de duraznos por métodos combinados. M.S. thesis, Universidad Nacional de Mar del Plata, Argentina.

Tilbury, R.H. 1976. the microbial stability of intermediate moisture foods with respect to yeasts. Ch. 11. In *Intermediate Moisture Foods*, R. Davies, G.G. Birch, and K.J. Parker (Ed.), p. 260. Applied Science, London.

Tilbury, R.H. 1980. Xerotolerant yeasts at high sugar concentrations. In *Microbial Growth and Survival in Extremes of Environment*, G.W. Gould and J.E.L. Corry (Ed.), p. 103. Academic Press, London.

Yarrow, D. 1984. *Zygosaccharomyces* Barker. Ch. III, Genus 33. In *The Yeasts. A Taxonomic Study*, N.J.W. Kreger-van Rij (Ed.), p. 449. Elsevier Science Publishers B.V., Amsterdam.

von Schelhorn, M. (1950). Untersuchungen Über den Verderb wasserarmer Lebensmittel durch osmophile Mikroorganismen. I. Verberb von Lebensmittel durch osmophile Hefen. Z. Lebensm. Unters. Forsch. 91: 117.

Warth, A.D. 1986. Effect of nutrients and pH on the resistance of *Zygosaccharomyces bailli* to benzoic acid. Intnal J. Food Microbiol. 3: 263.

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Van den Berg, C. and Bruin, S. 1981. Water activity and its estimation in food systems: theoretical aspects. In *Water Activity: Influences on Food Quality*, L.B. Rockland and G.F. Stewart (Ed.), p. 1. Academic Press Inc., London and New York.

Wilson, A.M., Work, T.M., Bushway, A.A., and Bushway R.J. 1981. HPLC determination of fructose, glucose and sucrose in potatoes. J. Food Sci. 46(1): 300–301.

Ms received 7/4/94; revised 10/16/94/ accepted 10/21/94.

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Riboflavin-Sensitized Photodynamic UV Spectrophotometry for Ascorbic Acid Assay in Beverages

M.Y. JUNG, S.K. KIM, and S.Y. KIM

ABSTRACT

Riboflavin-sensitized photodynamic ultraviolet spectrophotometric assay of various commercial beverages was carried out. The maximum concentration of ascorbic acid for obtaining a background correction (15 min illumination and pH 7.5) was 21 μg/mL ascorbic acid in the testing solution. The upper limit of the measurement range for a straight line in the calibration graph of standard ascorbic acid was 10 μg/mL, (working range 0–10 μg/mL). The results in repeatability of the method for ascorbic acid contents in commercial fruit juices and soft-drinks showed a maximum of 4% relative standard deviation. Recovery tests with known amounts of added ascorbic acid in different fruit juices and sports drink showed the recovery of added ascorbic acid was 97.5–102.3%. Indophenol, iodine and/or HPLC methods were used in parallel to ascertain the reliability of the proposed method. This type assay could be successfully applied to many commercial beverages for determination of ascorbic acid with good accuracy, precision and reliability.

Key Word: Ascorbic acid, photodynamic assay, riboflavin sensitized, fruit juices, sports drink

INTRODUCTION

ASCORBIC ACID occurs naturally in fruits and vegetables and is often added to fruit juices and sports drinks. Since it is easily oxidized during processing, handling and storage, the ascorbic acid content may be used as an index of quality. Commonly used methods for determining ascorbic acid in foods are indophenol method, iodine method and HPLC method. The indophenol method, based on titrimetry using the reducing power of ascorbic acid, can not be used for samples containing reductants or for colored samples. Iodine method has the disadvantage of redox reagent interference. High performance liquid chromatography (HPLC) has been used for ascorbic acid assay (Doner and Hicks, 1981; Rose and Nahrwold, 1981; Bradbury and Singh, 1986; Vanderslice and Higgs, 1993). However, the analytical conditions such as pre-treatment of samples, packing of columns and mobile phase differ with samples, so that the optimum conditions must be carefully tested for each assay.

Thus, a rapid, simple and reliable method is needed for determination of ascorbic acid. Direct UV spectrophotometry can provide such a fast and simple method (Fung and Luk, 1985a). However, the absorption of UV light by the sample matrix is the major problem with this method. Various background correction techniques such as thermal degradation, UV decomposition, enzymatic or metal catalytic oxidation, and alkaline treatment have been proposed to solve this problem. The thermal, UV, and metal catalytic decomposition of ascorbic acid was too slow to be used practically (Fung and Luk, 1985a). In the enzymatic technique, enzymes used were ascorbate oxidase (Tono and Fujita, 1981, 1982; Esaka et al., 1985), ascorbate peroxidase (Kelly and Latzko, 1980) and guaiacol peroxidase (Casella et al., 1989; Tsumura, et al., 1993). Although the enzymatic methods are simple, rapid and highly specific for ascorbic acid, the enzymes are relatively costly for routine analysis. Alkaline treatment is a rapid and simple method for determination of ascorbic acid (Fung and Luk, 1985a,b). However, this method is less sensitive because it is based on absorbance at 243 nm instead of 265 nm for quantitation of ascorbic acid. In addition, the calibration curve was not straight at low concentrations of ascorbic acid.

Ascorbic acid reportedly oxidized extremely fast in the presence of photosensitizers and light (Sattar et al., 1977; Bodannes and Chan, 1979; Chou and Khan, 1983; Rooney, 1983; Jung et al., 1994). Our previous results showed that 1.2×10^{-4}M ascorbic acid oxidized completely after a 12 min storage in the presence of 6.0 μg/mL riboflavin under 3300 lux fluorescent light because of the formation of singlet oxygen which is very reactive toward ascorbic acid (Jung et al., 1994). The bimolecular rate constant for reaction of ascorbic acid with singlet oxygen at pH 7.5 was 6.63×10^8 M ^{1}sec^{-1} (Jung et al., 1994). Thus, we expected that a rapid background correction for direct UV measurement of ascorbic acid could be achieved by photodynamic singlet oxygen formation. Thus, our objective was to establish a rapid riboflavin-sensitized photodynamic UV spectrophotometric assay for ascorbic acid in beverages.

MATERIALS & METHODS

Materials

L-ascorbic acid and riboflavin were purchased from Sigma Chemical Co. (St. Louis, MO). Serum bottles were obtained from Supelco Inc., (Bellefonte, PA). HPLC grade acetonitrile was obtained from Fisher Scientific Co. (Fair Lawn, NJ). Commercial beverages were purchased from local groceries.

Comparison of photochemical methods for making background corrections

To compare photochemical methods of riboflavin and methylene blue, and chemical H_2O_2-NaOCl treatment method for making background corrections, 21 μg/mL ascorbic acid solutions containing 6 μg/mL of riboflavin or methylene blue or containing 0.26M H_2O_2 and 0.072M NaOCl were prepared. The photochemical methods of riboflavin and methylene blue and chemical H_2O_2-NaOCl treatment method were well established ways to generate singlet oxygen. Samples (20 mL) treated with riboflavin and methylene blue were transferred into serum bottles and, then, placed, at room temperature (≈23°C) in a light storage box, described in detail previously (Fakourelis et al., 1987; Jung and Min, 1991; Jung et al., 1991). The light intensity at the sample level was 5500 lux. The sample prepared with H_2O_2-NaOCl was placed under dark. The content of ascorbic acid was determined by HPLC (Bradbury and Singh, 1986).

Photodynamic background correction

Riboflavin was chosen to achieve the background correction (sample blank) for a new direct UV analysis because of effectiveness in ascorbic acid destruction. To study the destruction of various concentrations of ascorbic acid, 9, 21, 35, 53 or 105 μg/mL ascorbic acid solutions containing 6 μg/mL riboflavin (pH 7.5) were prepared by addition of calculated amounts of ascorbic acid and riboflavin into 0.01M potassium buffer (pH 7.5). Prepared sample (20 mL) was transferred into serum bottles. The bottles were placed in the light storage box as described. The content of ascorbic acid during illumination was monitored by measuring absorbance at 265 nm using a spectrophotometer (Bodannes and Chan, 1979).

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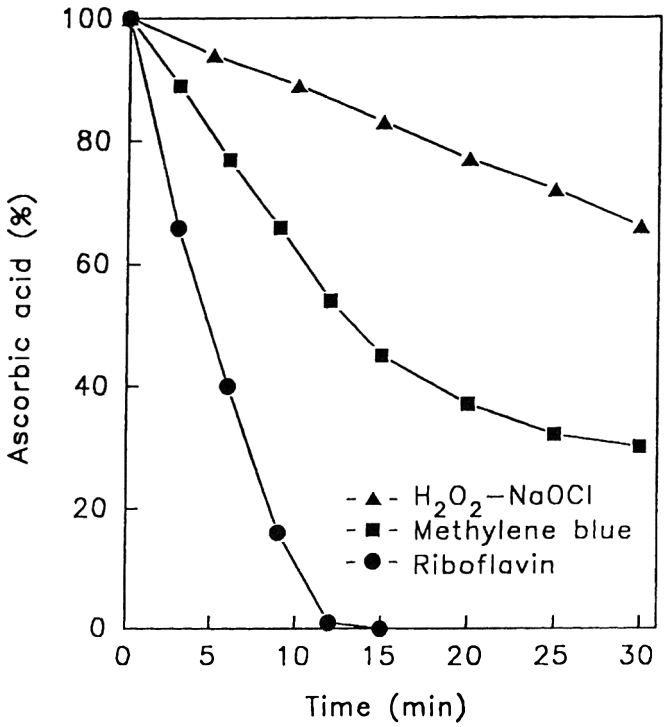

Fig. 1—Comparison of the destruction rates of ascorbic acid by photochemical methods of riboflavin and methylene blue, and chemical H_2O_2-NaOCl treatment method.

To study the destruction rates of ascorbic acid at different pH's, 21 μg/mL ascorbic acid solutions containing 6 μg/mL riboflavin (pH 7.5, 5.5 and 3.5) were prepared by addition of calculated amounts of ascorbic acid and riboflavin into 0.01 phosphate buffers of pH 7.5, 5.5 and 3.5, respectively. The content of ascorbic acid was determined by measuring absorbance of solutions at 265 nm for samples at pH 7.5 and 5.5, or at 245 nm for samples at pH 3.5 using a spectrophotometer.

Preparation of calibration graph

Tc prepare the calibration graph of standard ascorbic acid, 0–16 μg/mL of ascorbic acid solutions containing 6 μg/mL riboflavin (pH 7.5) were prepared by addition of calculated amounts of ascorbic acid and riboflavin into 0.01M phosphate buffer (pH 7.5). Six μg/mL riboflavin in 0.01M phosphate buffer (pH 7.5) was prepared and used as reagent blank. Differences in absorbances of standard solutions and reagent blanks measured at 265 nm were plotted vs concentrations of ascorbic acids.

Determination of ascorbic acid contents

To prepare a sample for analysis, 0.2–1.0 mL of juice or drink was transferred into a 250 mL beaker and ~ 40 mL or 90 mL of 0.01M phosphate buffer (pH 7.5) was added to get the approximate ascorbic acid concentration of <10 μg/mL. The pH of the diluted samples was checked. If the pH was not 7.5 because of addition of sample, it was adjusted to pH 7.5 with dilute NaOH solution, and the sample was then transferred into a 50 mL or a 100 mL volumetric flask. The beaker was washed with 0.01M phosphate buffer into the volumetric flask. Then, 0.5 mL or 1 mL of riboflavin stock solution (0.060g riboflavin in 100 mL of 0.01M phosphate buffer, pH 7.5) was added to the volumetric flask and then 0.01M phosphate buffer (pH 7.5) was added to the mark.

None of the commercial beverages needed to be pretreated for removal of solid materials. However, if the samples contained high concentrations of suspended solids interfering with the absorptivity at 265 nm we recommend centrifuging or filtering before UV analysis. Prepared sample (20 mL) was transferred into a 30 mL serum bottle and then placed in the light storage box for 15 min as described. We found that ascorbic acid in the prepared sample was completely destroyed after 15 min of light storage. Absorbances of samples before and after light storage were measured at 265 nm using a spectrophotometer. Differences in absorbance of samples before and after 15 min-illumination were used for calculation of ascorbic acid. The calibration graph prepared previously was used to determine the actual concentrations of ascorbic acid in the sample.

Method reliability was tested on, 12 commercial beverage samples by comparison with established methods. Three methods compared were the indophenol (AOAC, 1980), the iodine titration (Fung and Luk, 1985b) and HPLC method (Bradbury and Singh, 1986). The proposed direct UV method measures ascorbic acid but not dehydroascorbic acid because dehydroascorbic acid does not absorb light at 265 nm.

Indophenol method

The indophenol solution, 0.25 g/L of 2,6 dichloroindophenol, was standardized by titration with 2.0 mL of standard ascorbic acid solution and 5 mL of 3% metaphosphoric acid—8% acetic acid solution (HPO_3-HOAc) to the end point (a persistent rosy pink color). Consumption of the blank was determined by titrating indophenol solution with 7 mL of HPO_3-HOAC solution plus a given amount of water equivalent to the volume of indophenol solution used in the previous standardization titration. For the sample titration, 50 mL of sample was mixed with an equal volume of HPO_3-HOAc solution before filtering. A volume of filtrate equivalent to about 2 mg of ascorbic acid was then titrated with indophenol solution using the procedure described including titration of the blank.

Iodine method

The iodine solution (0.005M) was standardized in the usual way with 0.01M thiosulfate solution. For sample analysis, 5 mL of sample, 20 mL of water and 2 mL of 1% starch solution were titrated with standardized iodine solution.

HPLC method

A high performance liquid chromatograph (Model 45, Waters Associates, Milford, MA) was equipped with a UV detector (Waters Associate, Milford, MA) was used (Bradbury and Singh, 1986) for ascorbic acid determination. The column used was μ-Bondapak C-18 (30 cm × 3.9 mm, Waters Associates, Milford, MA). The mobile phase (flow rate 1.3 mL/min) was aqueous 0.005M KH_2PO_4 adjusted to pH 4.6 with dilute HCl and acetonitrile (30:70, v/v). Ten μL of sample was injected. The ascorbic acid was quantitated at 265 nm by the UV detector.

RESULTS & DISCUSSION

Background correction (sample blank)

Ascorbic acid is reportedly easily oxidized by the reaction with singlet oxygen (Bodannes and Chan, 1979; Chou and Khan, 1983; Rocney, 1983; Jung et al., 1994). Thus, we used photochemical methods of riboflavin and methylene blue and chemical H_2O_2-NaOCl treatment method to produce singlet oxygen. The relative rates of decomposition of ascorbic acid by photochemical methods of riboflavin and methylene blue, and chemical H_2O_2-NaOCl treatment method were compared (Fig. 1). The destruction of ascorbic acid by riboflavin sensitized photochemical method was fastest among the tested methods. After 15 min of illumination, 100% ascorbic acid was destroyed by riboflavin sensitized photooxidation. Thus it was selected for more detailed study for making the sample blank (background correction).

Effects of initial ascorbic acid concentration and pH

Riboflavin sensitized photodestruction of ascorbic acid was compared on different initial concentrations (9, 21, 35, 63 and 105 μg/mL) during light storage (Fig. 2). The destruction rate by riboflavin sensitized method increased rapidly with decreasing concentrations of ascorbic acid. As decomposition rate is dependent on initial concentration less time was required to destroy smaller amounts of ascorbic acid. The time required to destroy 9 μg/mL ascorbic acid was 12 min, and that to destroy 21 μg/mL ascorbic acid was 15 min under these conditions. The ascorbic acid destruction in the systems of 35 μg/mL, 63 μg/mL and 105 μg/mL ascorbic acid after 15 min-illumination were 98, 83 and 74%, respectively. This result suggested the samples should have <21 μg/mL ascorbic acid to be completely destroyed during a 15 min-illumination period.

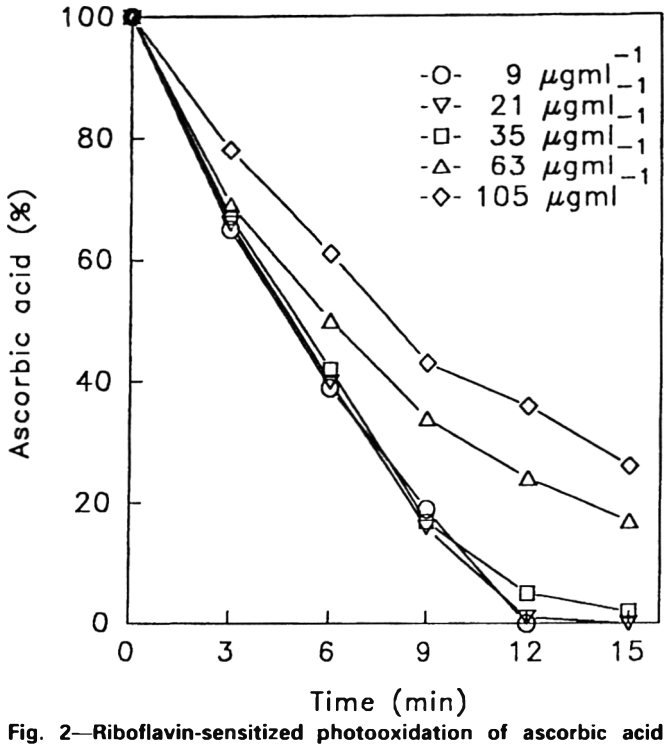

Fig. 2—Riboflavin-sensitized photooxidation of ascorbic acid with different initial concentration during light storage.

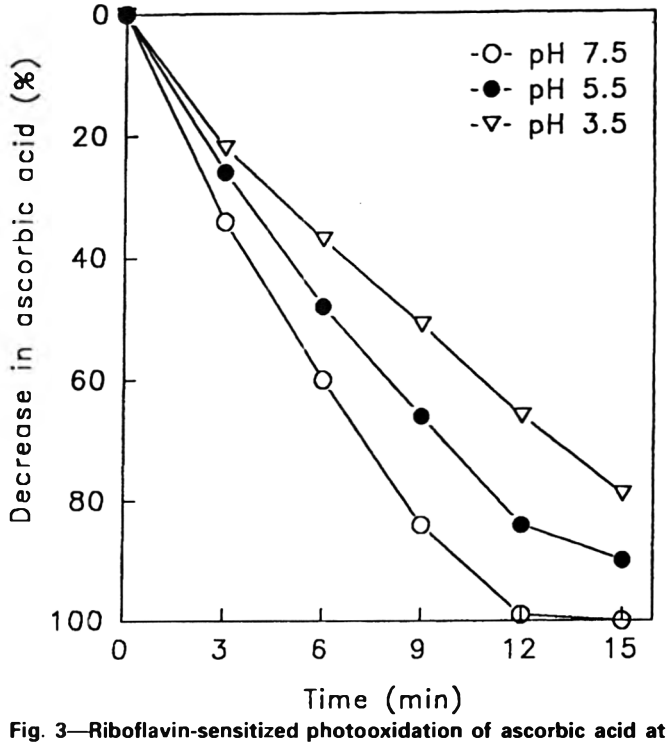

Fig. 3—Riboflavin-sensitized photooxidation of ascorbic acid at different pH during light storage.

Since ascorbic acid destruction by riboflavin was pH-dependent, the effects of pH (7.5, 5.5 and 3.5) on destruction of 21 μg/mL ascorbic acid solution containing 6 μg/mL riboflavin during light storage were studied (Fig. 3). Ascorbic acid destruction rate increased with increasing pH from 3.5 to 7.5. After 15 min light storage, ascorbic acid in solution at pH 7.5 was completely destroyed. That is, samples adjusted to pH 7.5 required 15 min to obtain the sample blank (background correction), and samples at lower pH required longer. As pH increased, the wavelength of maximal absorbance shifted from 245 to 265 nm, in confirmation of reported observations (Fung and Luk, 1985a). The absorption at 265 nm at pH 7.5 was much greater than that at 245nm at pH 3.5. Thus, the higher absorption at 265 nm at pH 7.5 increased precision of the assay. For analysis of ascorbic acid, pH 7.5 was used because of the higher absorption and faster destruction of ascorbic acid there. Destruction time of 15 min was selected for the optimal destruction of ascorbic acid.

Preparation of calibration graph

The limits of the working range were determined from the calibration graph of standard ascorbic acid (Fig. 4). The correlation coefficient (r) was 0.999. The maximum concentration of ascorbic acid was 10 μg/mL for a straight line relationship. That is, the upper limit of the measurement range was 10 μg/mL ascorbic acid. The molecular extinction coefficient for ascorbic acid was 14.3 mMcm^{-1}, which was almost the same as previously reported values at pH 7.0 (Kelly and Latzko, 1980; Tsumura, 1993). When the extinction coefficient of 14.3 mMcm^{-1} was used for calculation of ascorbic acid, the calibration graph was not needed as long as the concentration of ascorbic acid in the tested sample was <10 μg/mL.

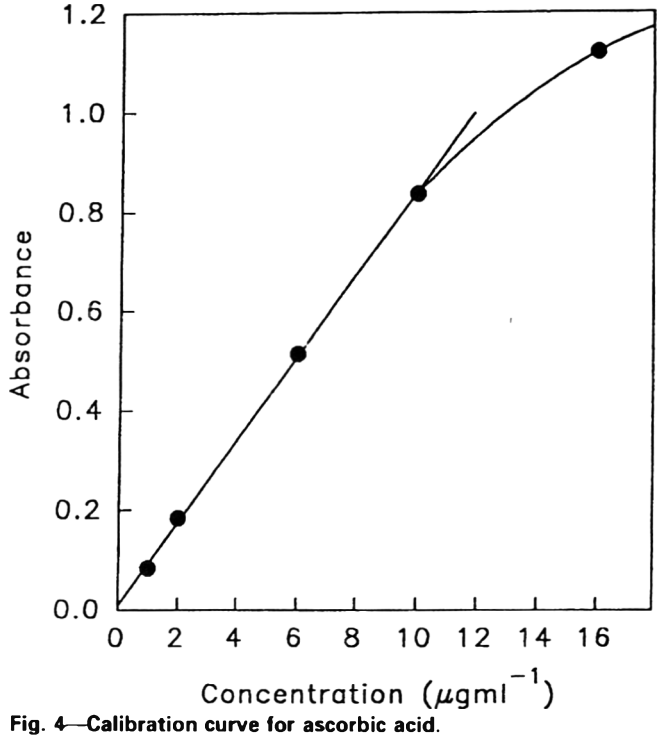

Fig. 4—Calibration curve for ascorbic acid.

Changes in spectra by photochemical background correction

The changes in spectra of orange juice and pineapple juice samples after photochemical background correction were compared (Fig. 5). Absorption at 265 nm in samples before the photodynamic step were due to both the ascorbic acid and unknown components in the samples. As previously mentioned, ascorbic acid in aqueous solution (pH 7.5) has absorption maximum at 265 nm. After the riboflavin-sensitized photodynamic background correction, considerable changes were observed in spectra around 265 nm (Fig. 5). The change in spectra around 265 nm by the photodynamic step seemed to be mostly due to the singlet oxygen oxidation of ascorbic acid. Ascorbic acid was extremely reactive to singlet oxygen (Bodannes and Chan, 1979; Chou and Khan, 1983; Rooney, 1983; Jung et al., 1994). The bimolecular reaction rate for reaction of ascorbic acid with singlet oxygen at pH 7.5 was $6.63 \times 10^8 M^{-1}sec^{-1}$ (Jung et al., 1994). We monitored ascorbic acid contents in the background corrected samples by the HPLC method, and found that no as-

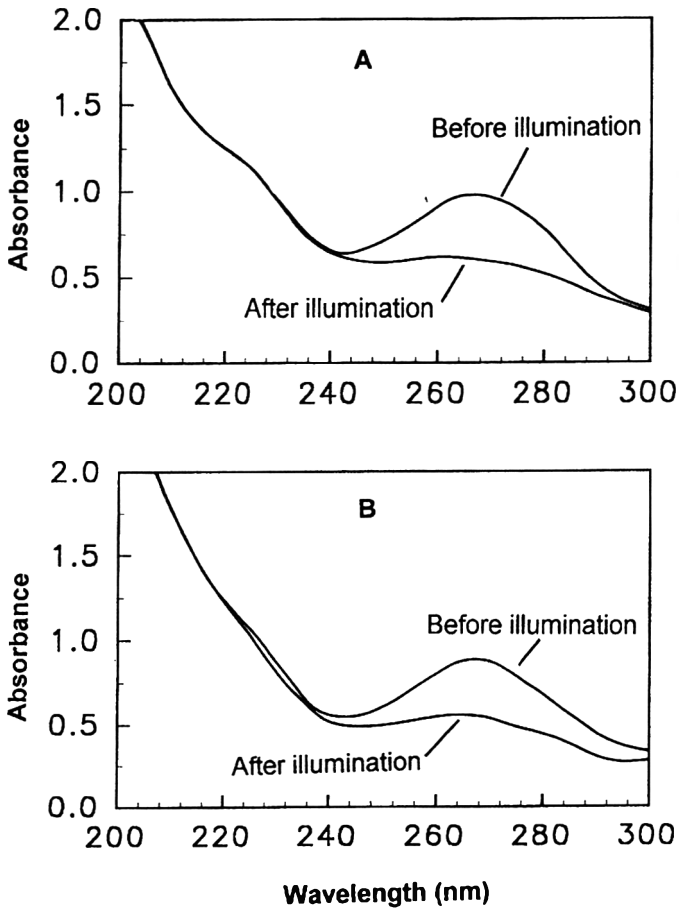

Fig. 5—Changes in spectra for A) orange juice and B) pineapple juice samples by the riboflavin-sensitized photodynamic background correction.

corbic acid remained in samples after the photodynamic background correction. Fifteen minutes of further illumination after the background correction did not induce further changes in spectra. This suggested that ascorbic acid was selectively destroyed by the formation of singlet oxygen under these conditions.

Table 1—Repeatability of methods for analysis of ascorbic acid contents in commercial beverages

	Beverages		
	Orange juice	Pine-apple juice	Sports drink
Ascorbic acid contents ($\mu g ml^{-1}$) by indophenol method	522.3	450.1	348.2
Ascorbic acid contents ($\mu g ml^{-1}$) by proposed method	529.2	456.7	337.4
No. of determinations	6	6	6
Relative standard deviation (%)	1.77	2.98	4.00

Table 2—Recovery with known amounts of added ascorbic acid in different beverages

Beverage	Ascorbic acid added (μgmL^{-1})	Ascorbic acid re-covered (μgmL^{-1})	Recovery (%)
Orange juice 1[a]	402.2	392.0±6.3	97.5
Orange juice 2	402.2	406.1±2.2	101.0
Pineapple juice	442.4	452.4±3.9	102.3
Sports drink	241.3	237.4±4.1	98.4

[a] Orange juices 1 and 2 are two different brands of juice.

Table 3—Comparison of results by proposed UV method with other analytical methods

	Ascorbic acid (μgmL^{-1})[a]			
Beverage	UV	IND	IOD	HPLC
Orange juice 1	529.2	522.3	—	529.1
Orange juice 2	319.6	332.0	—	—
Pineapple juice 1	456.7	450.1	—	463.6
Pineapple juice 2	256.3	249.1	—	—
Grapefruit juice 1	426.6	406.2	—	411.5
Grapefruit juice 2	315.7	294.5	—	—
Acerola drink	375.0	408.2	—	424.9
Sports drink	337.4	348.2	—	—
Vegetable juice cocktail	245.4	186.7	—	—
Tomato juice	120.9	—	94.4	120.3
Grape juice	80.2	—	78.5	90.0
Apple juice	80.2	—	57.2	—

[a] UV, IND, IOD and HPLC represent the proposed UV spectrophotometry, indophenol, iodine and high performance chromatographic methods, respectively.

Ascorbic acid content in commercial beverages

Repeatability of the method for analysis of ascorbic acid contents in selected commercial beverage was tested (Table 1). The results show <4% relative standard deviation for three samples studied. The riboflavin sensitized photochemical method for ascorbic acid contents in commercial beverages gave precise results in analyzing real fruit juices and sport drink.

The recovery test results with known amounts of added ascorbic acid in four different commercial beverages were compared (Table 2). Almost 100% recovery was shown with no observed significant deviation for any of the samples tested.

To test the reliability of the method, 12 commercial beverage samples were collected, and parallel determinations using well established methods and the proposed procedure were compared. The indophenol method has interference from colored samples (especially red), thus, for some samples, the second iodine titration method, which has less interference form colored solutions, was used. However, redox impurities can cause problems in that analysis. The third HPLC method for determination of ascorbic acid was used, but with interfering constituents, separation of ascorbic acid was difficult in some beverages.

The results with the proposed method, and the three established methods were compared (Table 3). The concentration of ascorbic acid varied greatly among the different samples. The proposed method had a distinct advantage over the AOAC indophenol method. The proposed method could measure ascorbic acid in some colored samples such as tomato juice and grape juice. Ascorbic acid contents in tomato juice and grape juice could not be measured by AOAC indophenol method because of color interferences. Among the 12 tested samples, the ascorbic acid contents in 9 samples were successfully determined by the proposed method. However, considerable differences were observed between references and the proposed method for acerola drink, vegetable juice cocktail and apple juice. Reasons for these differences are uncertain.

REFERENCES

AOAC 1980. *Official Methods of Analysis*, 13th ed. W. Horwitz (Ed.), p. 746. Association of Official Analytical Chemists, Washington, DC.

Bodannes, R.S. and Chan, P.C. 1979. Ascorbic acid as a scavenger of singlet oxygen. FEBS Letters. 105: 195–196.

Bradbury, J.H. and Singh, U. 1986. Ascorbic acid and dehydroascorbic acid content of tropica. root crops from the south pacific. J. Food Sci. 51(4): 975–978, 987.

Casella, L., Gullotti M., Marchesini, A., and Petrarulo, M. 1989. Rapid enzymatic method for vitamin C assay in fruits and vegetables using peroxidase. J. Food Sci. 54: 374–378.

Chou, P. and Khan, A.U. 1983. L-ascorbic acid quenching of singlet oxygen delta molecular oxygen in aqueous media; generalized antioxidant property of vitamin C. Biochem. Biophys. Res. Commun. 115: 932–936.

Doner, L.W. and Hicks, K.B. 1981. High-performance liquid chromatographic separation of ascorbic acid, erythorbic acid, dehydroascorbic acid, dehydroerythorbic acid, diketogluonic acid, and diketogluconic acid. Anal. Biochem. 115: 225–230.

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Consumer Age Affects Response to Sensory Characteristics of a Cherry Flavored Beverage

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ABSTRACT

The sensory responses of a young adult population were compared to one over age 60. Using a central composite design, sweetness and flavor intensity, flavor quality, overall acceptability, perceived thirst quenching and flavor identification were evaluated using an artificially flavored cherry beverage varying in sucrose, flavor, and color. The mean data were modeled to a response surface as a function of sucrose, color, and flavor. In both populations the measures were responsive to factors manipulated in the design. Color had specific unique effects on overall acceptance, flavor quality, and intensity in each of the populations. The older population was more sensitive to visual cues and less sensitive to changes in flavor concentration.

Key Words: cherry beverage, sensory, color, flavor, consumer age

INTRODUCTION

COLOR has a strong effect on the way we perceive everything around us. The Good Housekeeping Institute (1984) has reported that 29.5% of their readership rated "good appearance or good color" second only to "freshness" (52.5%) and well above taste (23.5%), texture (10.3%), and smell (5.0%) when asked to rank a list of food quality attributes. Since freshness was considered more of a quality than a sensory factor such results indicate that color is very important in the acceptability of a food product.

Color influences the identification of flavors. DuBose et al. (1980) demonstrated that the number of correct flavor identifications decreased sharply when an inappropriate color was used with a flavored beverage. Hall (1958) demonstrated the same effect using colored sherbets. Studies by Gifford and Clydesdale (1986) and Gifford et al. (1987) evaluated the effects of color on salt perception. Roth et al. (1988) studied the effect of color on sweetness perception, and Tuorila-Ollikainen et al. (1984) evaluated the effects of color on flavor in flavored and unflavored beverages.

One aspect that has been lacking in most studies on color is an evaluation of its effect on the perception of other sensory characteristics as related to subjects' age (Clydesdale, 1993). As people grow older, vision and hearing decline. A notable decline also occurs in olfactory sensitivity and a more modest decline in taste sensitivity (Cowart, 1989). Schiffman and Warwick (1989) indicated that the thresholds for many odors were often 12 times higher in the elderly as compared to younger persons. No attempts have been made to compensate for such losses in acuity.

Schiffman and Warwick (1989) found that the elderly preferred 19 out of 20 flavor enhanced samples over unenhanced counterparts. In evaluating taste sensitivity to sweet, sour, bitter, and salt, Cooper et al. (1959) found little change in sensitivity in subjects up to age 50, but after the mid 50s a sharp decline occurred in sensitivity to all four tastes.

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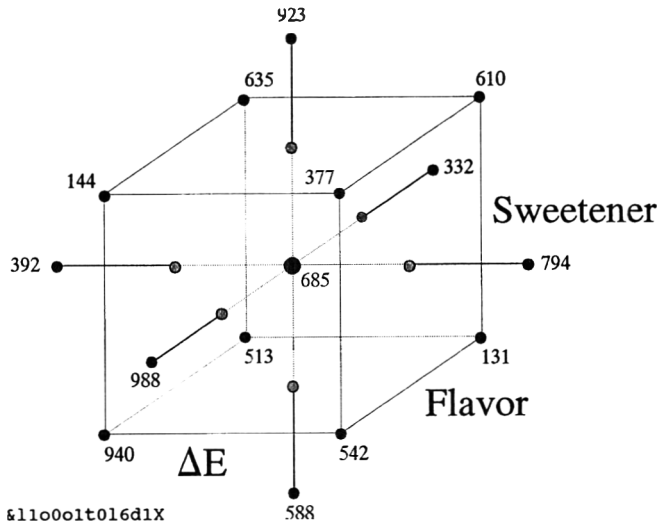

Fig. 1—Relative position of samples in three-dimensional space with each outside point and the four corners of the cube all touching the outside of a sphere which could be drawn around the cube.

Response to thirst changes with age. Rolls (1989) studying fluid deprived subjects found responses of the elderly were very different from younger subjects. An older individual reported no feelings of thirst at all. Fluid intake is critical for everyone, but becomes more critical when sensitivity to the thirst stimulus is low.

Our objective was to determine differences in sensory responses between young adults, (ages 18–22) and an older population, (ages 60–75) to varying concentrations of color, flavor, and sucrose in an artificially flavored cherry beverage. Subjects evaluated perceived sweetness, flavor intensity, flavor quality, overall acceptability, and perceived thirst quenching.

MATERIALS & METHODS

TWO SEPARATE PANELS of untrained subjects were used: one was 69 students, ages 18–22, from an undergraduate nonmajor food science class at the University of Massachusetts, and the other was 55 persons, ages 60–75. The older population was recruited from a mailing list obtained from the town of Amherst, Massachusetts. A letter was mailed to a random sample from this list, followed by a telephone call indicating the purpose of the research. Individuals who knew they were color blind did not participate.

Sample variations were developed using a central composite rotatable design (CCD) (Meilgaard et al., 1991; Gacula and Singh, 1984; Box et al., 1978) (see Fig. 1). The design was composed of a 2^3 factorial (the eight corners of the cube), a center point (#685) and "axial" points (emanating out from the center point). The design yields good resolution (i.e., five levels across each of three ingredient factors) in an efficient manner (i.e., only 15 samples were required as compared to 27 samples in a traditional 3^3 factorial). In the design, the center point was set as the standard (0) and each of the remaining 14 samples was formulated to contain more or less of the three variables under study, color, flavor and sucrose.

The composite rotatable design required that the distance, α, from the center point (standard) to the axial point (extreme samples) be equal to $Nc^{1/4}$. In the second order design with 3 variables N = 8 and $\alpha = 8^{1/4} = 1.68$ (Gacula and Singh, 1984).

Thus, formulations were developed by placing the standard beverage as the center point and the extreme samples were chosen to represent concentrations of variables which were assumed to be on the fringe of acceptability by scientists at Kraft Foods who prepared the uncolored samples. These were then given a value of ±1.68 from the standard at 0. The less extreme samples were formulated at ±1 in relation to the standard at zero and the extreme samples at 1.68 (Table 1). Actual concentrations of sucrose and flavor were proprietary information (Kraft Foods). Coded units (0, ±1, ±1.68) were used to define levels of sucrose and flavor. However, colorants were added in the laboratory and blends were developed (Table 2).

Differences in colorant concentration did not correspond to differences in color space nor to visual assessments of color differences (Francis and Clydesdale, 1975). Therefore it was necessary to combine blends (from Table 2) to produce samples used (Table 3) which varied from yellow to red and had color differences of 7.9 and 13.3 ΔE units from the standard, corresponding to the ratio of 1:1.68 in accordance with the design. Tristimulus values, L, a and b, of each sample were measured on a Hunter LabScan II Colorimeter (Hunter Associates Laboratory Inc., Restin, VA) and the distance (ΔE) from the standard was calculated from the equation $\Delta E = (\Delta L^2 + \Delta a^2 + \Delta b^2)^{1/2}$ (Francis and Clydesdale, 1975).

Samples were assigned 3-digit random codes and presented such that each was seen in each position at least once. Presentations were rotated and balanced to the extent allowed by the number of subjects used. Specific rotation employed was such that each sample was rotated through all presentation positions with every 30 subjects. Thus, the student population (69 subjects) rotated more than twice through the complete order of samples and the older population (55 subjects) rotated through the design slightly less. Sample presentation was rotated and balanced as close as possible, given the ending sample size, to account for position (i.e., first, second, third, etc.) and sequential carry over effects (i.e., mediocre samples preceded or followed by more or less acceptable samples) (Meilgaard et al., 1991; Box et al., 1978).

Dry powdered cherry flavored beverage mixes for each variable except color were produced by General Foods to insure commercially acceptable quality. Packets containing the specified flavor and sucrose concentrations were designed to make 1860 mL of beverage. To make up this volume, 1822 mL of double distilled deionized water was used and a total of 38.0 mL of colorant blend added. Table 3 shows beverage code numbers, the coded units, quantity of blends added and ΔE values. Each sample was made 24 hr prior to testing and stored at 1.7°C until use.

Eight samples, containing 100 mL each in ≈265 mL clear plastic tumblers covered by paper boxes, were presented to each subject on a tray on day 1 under standard tungsten room lighting. The second eight samples were presented on day 2 with one sample overlapping for continuity under the same conditions. That is, for each subject, the last sample evaluated on day 1 was the first sample on day 2. Subjects were asked to use a 9-point rating scale and ratings for the first sample on day 2 were discarded from the analysis. Each sample was evaluated only once and rated for sweetness intensity, flavor intensity, overall acceptability, perceived ability to quench thirst, and flavor identification.

DATA ANALYSIS

DATA WERE SUBJECTED to analysis of variance using age, color, flavor and sucrose levels as independent variables. Subsequently, data were fitted to a regression equation for the younger and older groups separately using SAS STAT (SAS Institute Inc., Cary, NC). Regression equations were then plotted as three dimensional surface plots (SAS GRAPH) to observe effects each variable had within each population and between the two populations. Following regression analysis data were plotted with dependent variables on the Y axis and sucrose level (x axis) and color (z axis) (expressed in coded units).

RESULTS & DISCUSSION

COEFFICIENTS OF DETERMINATION from predicted regression models relating independent and dependent variables varied from 0.895 to 0.977 indicating that regression models adequately fit the data accounting for 89 to 98 percent of variance.

Sweetness perception

As expected both the younger and older populations perceived increasing sweetness with increasing sucrose concentration (Fig. 2). However, a difference (p = 0.0160) occurred in the way each age group responded to increases in sucrose level. Perception of sweetness of young adults increased more (Fig. 2a) than older subjects (Fig. 2b) with increasing sucrose concentrations. The muted response of older subjects was consistent with a decrease in gustatory sensitivity noted by Schiffman (1977) and Cooper et al. (1959).

A decrease in flavorant concentration from the high to low level caused a uniform decrease in perceived sweetness for both older (p = 0.0727) and younger (0.0519) subjects. Colorant concentration, however, did not affect sweetness perception in either population (Fig. 2a and b). Results indicated that there may have been an upward trend with increasing color in the older population at the lower sucrose level.

Table 1—Relative concentrations, expressed as coded units, independent variables within each sample evaluated

Sample no.	Coded units		
	Sucrose	Cherry flavorant	Colorant[a]
923	+1.68	0[b]	0[b]
144	+1.0	−1.0	−1.0
377	+1.0	−1.0	+1.0
635	+1.0	+1.0	−1.0
610	+1.0	+1.0	+1.0
983	0[b]	−1.68	0[b]
392	0[b]	0[b]	−1.68
685	0[b]	0[b]	0[b]
794	0[b]	0[b]	+1.68
332	0[b]	+1.68	0[b]
940	−1.0	−1.0	−1.0
542	−1.0	−1.0	+1.0
513	−1.0	+1.0	−1.0
131	−1.0	+1.0	+1.0
588	−1.68	0[b]	0[b]

[a] Variations from the standard colorant concentration were expressed as color difference ratios (ΔE). Actual amounts of colorant used may be deduced from Table 2 and 3.

[b] 0 represents the concentration of the standard formulation. Variations from the standard were in the ratio of ±1 or ±1.68.

Table 2—Composition of blends used with red dye #40 (lot 588-T) and yellow dye #5 (lot 583-T, Warner Jenkinson Co, St. Louis, MO) mixed with double distilled deionized water (dd H_2O)

Blend #	g red dye #40	g yellow dye #5	mL dd H_2O
1	2.0	0.0	200
2	0.8	0.0	200
3	0.2	0.0	200
4	0.088	0.0	200
5	0.050	0.0	200
6	0.0	1.6	200
7	0.0	2.0	200
8	0.0	3.0	200

Table 3—Coded units, dyes added, and resulting color differences (ΔE) from the standard formulation

Bev	Coded units	Red blend #	Yellow blend #	ΔE[a]
1	−1.68	19 mL blend 5	19 mL blend 8	13.3
2	−1.0	19 mL blend 4	19 mL blend 8	7.9
3	−1.0	19 mL blend 4	19 mL blend 8	7.9
4	−1.0	19 mL blend 4	19 mL blend 8	7.9
5	−1.0	19 mL blend 4	19 mL blend 8	7.9
6	0	19 mL blend 3	19 mL blend 7	0
7	0	19 mL blend 3	19 mL blend 7	0
8	0	19 mL blend 3	19 mL blend 7	0
9	0	19 mL blend 3	19 mL blend 7	0
10	0	19 mL blend 3	19 mL blend 7	0
11	+1.0	19 mL blend 2	19 mL blend 6	7.9
12	+1.0	19 mL blend 2	19 mL blend 6	7.9
13	+1.0	19 mL blend 2	19 mL blend 6	7.9
14	+1.0	19 mL blend 2	19 mL blend 6	7.9
15	+1.68	37.2 mL blend 1	0 mL blend	13.3

[a] ΔE is a vector which indicates distance but not direction. The values obtained are in the ratio of 0:1:1.68 to conform with Table 1.

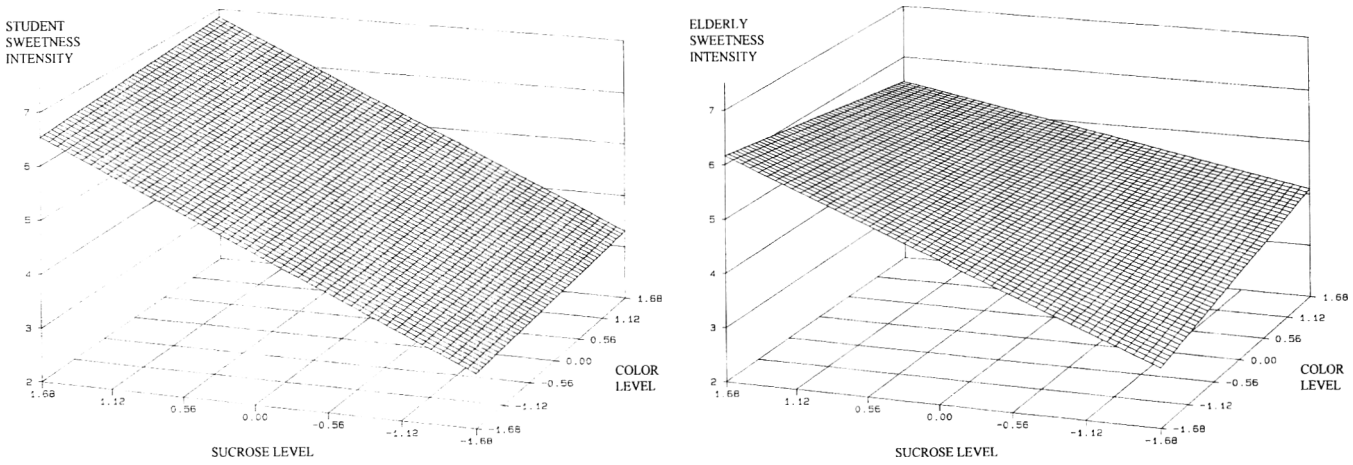

Fig. 2—Ratings for perceived sweetness intensity evaluated by (a) younger and (b) older subjects as related to sucrose concentration (coded units) and colorant level. Flavor was held constant at the high level. The fitted regression equation for (a) is Sweetness Intensity = 4.7282 + 0.0404 × sucrose level + 0.0384 × colorant level + 0.0038 × flavor level + 0.0002 × sucrose level × flavor level and for (b) is Sweetness Intensity = 4.5449 + 0.0321 × sucrose level + 0.0226 × colorant level + 0.0035 × flavor level − 0.0030 × sucrose level × colorant level.

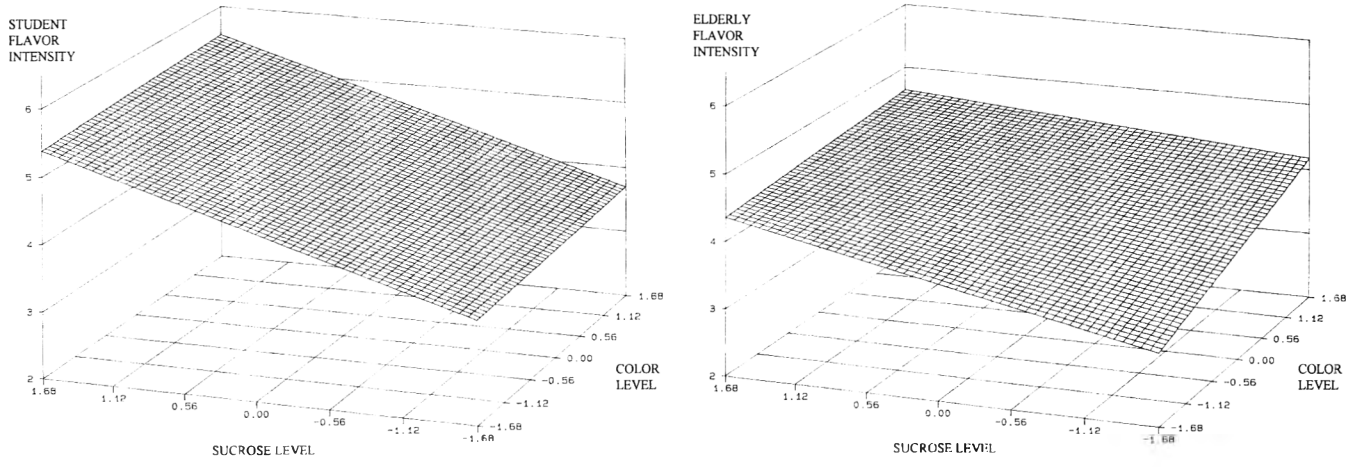

Fig. 3—Ratings for perceived flavor intensity evaluated by (a) younger and (b) older subjects related to sucrose level (coded units) and colorant level. Flavor was held constant at the high level. The fitted regression equation for (a) is Flavor Intensity = 4.3564 + 0.0265 × sucrose level + 0.0213 × colorant level + 0.0036 × flavor level and for (b) is Flavor Intensity = 3.8535 + 0.0126 × sucrose level + 0.0871 × colorant level + 0.0041 × flavor level − 0.0015 × sucrose level × colorant level.

Flavor intensity

Flavor intensity was affected in both populations over the range of sucrose concentrations, but effects on older subjects were somewhat muted and different ($p = 0.0001$) from younger subjects. The slope of the response curve for flavor intensity was much steeper for younger subjects (Fig. 3a) than for older subjects (Fig. 3b). This was not true for color where the effect on flavor intensity for the older subjects (Fig. 3b) was a ($p = 0.20$) steeper response than for younger subjects (Fig. 3a) as color shifted from yellow to red. The effects of color on flavor intensity were consistent with other reported results (Clydesdale et al., 1992). Of particular interest was the greater emphasis apparently placed on visual cues by older subjects as compared to younger subjects. A change in flavorant concentration from lowest to highest level produced a change in flavor intensity (young, $p = 0.0526$ and old, $p = 0.0327$) which was not different between the two populations.

Flavor quality

Subjects also evaluated "flavor quality" as well as flavor intensity. "Flavor quality" was defined as the subjects perception of trueness of flavor. We were not certain how subjects interpreted this meaning.

There was a difference ($p = 0.0109$) in perception of flavor quality by the two populations. Increasing sucrose concentration affected flavor quality response in both populations and the older subjects had a more muted response to changing sucrose concentrations (Fig. 4b) than the younger subjects (Fig. 4a). As color shifted from yellow to red it had an effect on assessment of flavor quality by both younger ($p = 0.0256$) and older ($p = 0.0693$) subjects. Note however, that a shift from lowest to highest flavorant level did not affect the older subjects' perception of flavor quality but increased it ($p = 0.0645$) in the younger group.

Overall acceptability

The apparent decreased importance of flavorant concentration for older subjects was apparently confirmed by the fact that it did not affect overall acceptability scores although sucrose and color did. Also, all three factors affected overall acceptability in younger subjects (Table 4) showing that the lack of effect of flavorant concentration was specific to older subjects.

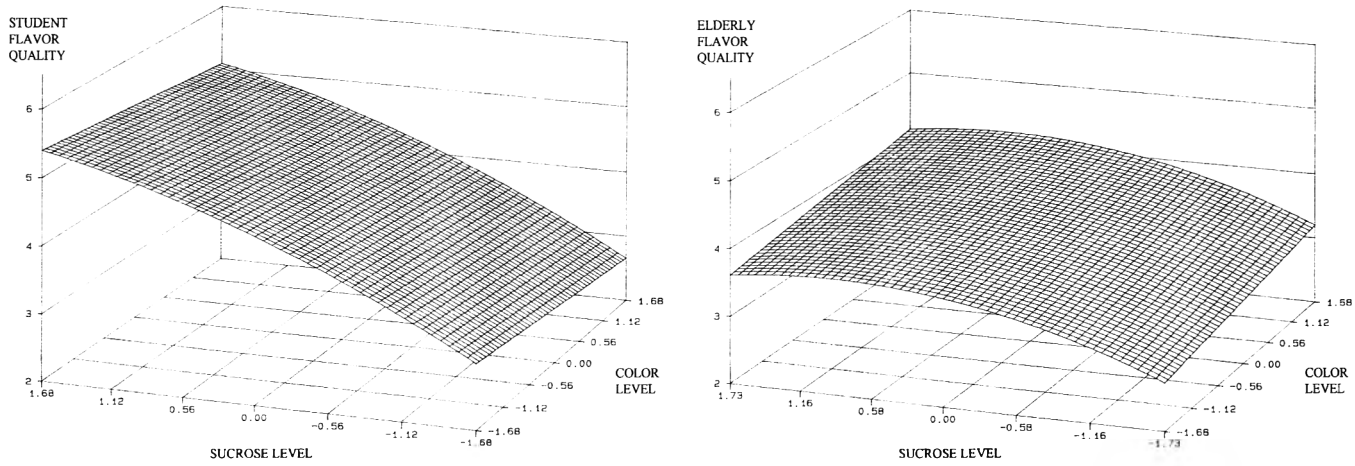

Fig. 4—Ratings for perceived flavor quality evaluated by (a) younger and (b) older subjects and colorant level as related to sucrose level. The fitted regression equation for (a) is Flavor Quality = 4.1300 + 0.0255 × sucrose level + 0.0559 × colorant level + 0.0036 × flavor level + 0.0002 × sucrose level × flavor level − 0.0002 × sucrose level × sucrose level − 0.0019 × flavor level × colorant level and for (b) is Flavor Quality = 3.8822 + 0.0187 × sucrose level + 0.0625 × colorant level + 0.0009 × flavor level + 0.0001 × sucrose level × flavor level − 0.0004 × sucrose level × sucrose level. Flavor was held constant at the high level.

Table 4—Effects of a change in sucrose concentration, colorant, or flavorant, noted as a p value, in an artificially flavored cherry beverage on the sensory perceptions of young adults (Y), ages 18–22, and an older population (O), ages 60–75

	Sensory perceptions[a]				
	Sweetness intensity	Flavor intensity	Flavor quality	Overall acceptability	Ability to quench thirst
Group					
			Effect of sucrose		
Y	0.0001	0.0001	0.0001	0.0001	0.0020
O	0.0001	0.0001	0.0001	0.0001	0.0121
			Effect of colorant		
Y	NS	NS	0.0256	0.0001	NS
O	NS	0.0120	0.0693	0.0409	NS
			Effect of flavorant		
Y	0.0519	0.0526	0.0645	0.0033	NS
O	0.0727	0.0327	NS	NS	NS

[a] significant positive effect is noted by a numerical p value while NS denotes no significant effect.

Table 5—Percent of responses to the question "what flavor would you say this beverage is?" for each flavor category of a young adult population (Y) age 18 to 22 (N=69), and an older population (O) age 60 to 75 (N=55) evaluting an artificially flavored cherry beverage colored from red to yellow

Bev #		Cherry	Fruit punch	Orange	No ans.	Other reds	Other
794	Y	48	7	7	19	12	4
	O	40	11	7	7	29	5
131	Y	38	12	19	16	7	7
	O	25	7	29	16	13	9
377	Y	45	7	19	17	12	4
	O	24	13	29	31	11	5
542	Y	29	9	19	25	12	7
	O	18	7	33	27	7	7
610	Y	51	6	9	19	13	3
	O	24	15	35	15	7	5
332	Y	22	9	30	25	7	7
	O	9	16	42	25	2	5
588	Y	16	7	36	29	4	7
	O	9	9	45	25	5	5
685	Y	19	6	42	23	4	6
	O	13	9	49	24	2	4
923	Y	20	10	38	12	13	7
	O	11	9	45	22	4	9
983	Y	22	7	38	23	6	4
	O	5	9	44	27	5	9
144	Y	12	4	46	19	7	12
	O	11	7	47	20	4	11
513	Y	17	3	36	29	9	6
	O	11	9	44	16	4	16
635	Y	19	3	42	16	13	7
	O	11	9	42	25	5	7
940	Y	14	1	49	25	3	7
	O	11	11	47	16	4	11
392	Y	23	7	38	14	7	10
	O	13	11	44	24	4	5

Thirst quenching

Perceived thirst quenching was not designed to determine a "real" effect but rather a perceived effect. That is, we did not try to measure the amount of beverage required to satisfy a thirsty subject but asked subject's opinions on which beverage they thought would best quench thirst. Such information may have some value in marketing but has little application in physiology. Nevertheless results were interesting since increasing sucrose only, and not flavor nor color, increased the perception of thirst quenching in both age groups (Table 4). However, the effects of sucrose, were muted in the elderly across the range of sucrose concentrations.

Effects of sucrose, flavor, and color

Useful information may be revealed by reviewing the data, grouped according to independent variables, sucrose, flavorant and colorant concentration (Table 4). Sucrose concentration, as opposed to colorant and flavorant, was the only dependent variable which had an effect on all attributes. Color affected several of the attributes, but contrary to other reported results (Clydesdale et al., 1992: Kostyla and Clydesdale, 1978: Maga, 1974; Pangborn, 1960), a change in color concentration had no effect on perceived sweetness intensity for either population. Color had no effect on perceived flavor intensity by the student population, but there was an increase in perceived flavor intensity of beverages by the older group. Similar results were noted by Schiffman and Pasternack (1979), where the absence of color greatly reduced or eliminated the ability to identify a flavor. Tepper (1993) reported that older subjects were more strongly influenced than younger ones by an off-color in orange juice. Apparently from our results, the older subjects depended more on visual cues to determine characteristics of the products. For both populations, an increase in color resulted in an increase in perceived flavor quality (Table 4) and acceptability as previously noted by Clydescale et al., 1992; Pangborn, 1960, and others. Color had no effect on perception of thirst quenching in either group.

An increase in flavor of 50% from the lowest to highest level caused an increase in perceived sweetness and flavor intensity for both populations (Table 4). However, the older adults did

not perceive an increase in flavor quality whereas the younger ones did. Similar results were found with regard to overall acceptability, where flavor was a significant factor when increased 50% for the younger, but not for the older, subjects. Perceived ability to quench thirst was not affected by flavor concentration in either population (Table 4).

The overall effects of color on flavor identification in all the samples were compared (Table 5). Although this was a peripheral study the results were interesting considering that the samples differed only in concentration of flavor, sucrose and color. As color changed from red through orange to yellow (Table 5) the number of correct responses identifying the flavor as cherry decreased. Identification as orange flavor increased along with the inability to identify the flavor (no answer) in both populations. The categories of "fruit punch," "other red flavors" and "other" changed randomly with no apparent trends. These results confirm others such as DuBose et al. (1980) where color was a critical component of flavor identification. However, in our results the younger population gave a greater number of correct responses than older subjects, likely reflecting decreasing olfaction sensitivity with age and increasing reliance on visual cues.

From these results, and others, sensory perception is apparently age dependent and color may become more influential in perception of other sensory characteristics, with age. Color is important for more than appearance. Color obviously provides cues which cause interactions with perceptions of other sensory characteristics (as do taste and flavor).

More research is needed to quantify such interactions. Consideration should be given to formulating foods with flavor and/or color and/or taste enhancement to provide foods that may be more appealing to older consumers.

REFERENCES

Box, G.E., Hunter, W.G., and Hunter, J.S. 1978. *Statistics for Experimenters*, p. 510–538. John Wiley & Sons, New York.

Clydesdale, F.M. 1993. Color as a factor in food choice. Crit. Rev. Food Sci. Nutr. 33: 83–101.

Clydesdale, F.M., Gover, R., Philipsen, D.H., and Fugardi, C. 1992. The effect of color on thirst quenching, sweetness, acceptability, and flavor intensity in fruit punch flavored beverages. J. Food Quality 15: 19–38.

Cooper, R.M., Bilash, I., and Zubek, J.P. 1959. The effect of age on taste sensitivity. Journal of Gerontology. 14: 56–58.

Cowart, B.J. 1989. Relationship between taste and smell across the Adult Life Span. In *Nutrition and the Chemical Senses in Aging: Recent Advances and Current Research Needs*, C. Murphy, W.S. Cain, and D.M. Hegsted (Ed.), p. 39–55. New York Academy of Sciences, New York.

DuBose, C.N., Cardello, A.V., and Maller, O. 1980. Effects of colorants and flavorants on identification of perceived flavor intensity, and hedonic quality of fruit flavored beverages and cake. J. Food Sci. 45: 1393–1399, 1415.

Gacula, M.C. Jr. and Singh, J. 1984. *Statistical Methods in Food and Consumer Research*. Academic Press, Inc., Orlando, FL.

Francis, F.J. and Clydesdale, F.M. 1975. *Food Colorimetry: Theory and Applications*. AVI Publ. Co., Westport, CT.

Gifford, S.R., and Clydesdale, F.M. 1986. The psychophysical relationship between color and sodium chloride concentrations in model systems. J. Food Prot. 49: 977–982.

Gifford, S.R., Clydesdale, F.M., and Damon, R.A., Jr. 1987. The psychophysical relationship between color and salt concentration in chicken flavored broths. J. Sensory Studies 2: 137–147.

Good Housekeeping. 1984. Consumer Food and Nutrition Study a Good Housekeeping Institute Report. May. Consumer Research Department. Good Housekeeping Institute, New York.

Hall, R.L. 1958. Flavor study approaches at McCormick and Co., Inc. In *Flavor Research and Food Acceptance*. Arthur D. Little, Inc., (Ed.). Rheinhold, New York.

Kostyla, A.S. and Clydesdale, F.M. 1978. The psychophysical relationship between color and flavor. CRC Crit. Rev. Food Sci. Nutr. 10: 303–321.

Maga, J.A. 1974. Influence of color on taste thresholds. Chemical Senses & Flavor 1: 115–119.

Meilgaard, M., Civille, G.V., and Carr, B.T. 1991. *Sensory Evaluation Techniques*. CRC Press Inc., Boston, MA.

Pangborn, R.M. 1960. Influence of color on the discrimination of sweetness. Am. J. of Psych. 73: 229–239.

Rolls, B.T. 1989. Regulation of fluid intake in the elderly. In: Murphy, C., Cain, W.S., and Hegsted, D.M., Eds. *Nutrition and the Chemical Senses in Aging: Recent Advances and Current Research Needs*. C. Murphy, W.S. Cain, and D.M. Hegsted (Ed.), p. 217–225. New York Academy of Sciences, New York.

Roth, H.A., Radle, L., Gifford, S.R., and Clydesdale, F.M. 1988. Psychophysical relationships between perceived sweetness and color in lemon and lime flavored beverages. J. Food Sci. 53: 1116–1119, 1162.

Schiffman, S.S. 1977. Food recognition in the elderly. Journal of Gerontology. 32: 586–592.

Schiffman, S.S. and Pasternak, M. 1979. Decreased discrimination of food odors in the elderly. J. Gerontology 34: 73–79.

Schiffman, S.S. and Warwick, Z.S. 1989. Use of flavor amplified foods to improve nutritional status in elderly persons. In *Nutrition and the Chemical Senses in Aging: Recent Advances and Current Research Needs*. C. Murphy, W.S. Cain, and D.M. Hegsted (Ed.), p. 261–276. New York Academy of Sciences, New York.

Tepper, B.J. 1993. Effects of a slight color variation on consumer acceptance of orange juice. J. Sensory Studies 8: 145–154.

Tuorila-Ollikainen, H., Mahlamaki-Kultanen, S., and Kurkela, R. 1984. Relative importance of color, fruity flavor, and sweetness in overall liking of soft drinks. J. Food Sci. 49: 1598–1600, 1603.

Ms received 5/21/94; revised 10/14/94; accepted 11/25/94.

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Esaka, M., Suzuki, K., and Kubota, K. 1985. Determination method for L-ascorbic acid in foods with immobilized ascorbate oxidase. Agric. Biol. Chem. 49: 2955–2960.

Fakourelis, N., Lee, E.C., and Min, D.B. 1987. Effects of chlorophyll and β-carotene on the oxidation stability of olive oil. J. Food Sci. 52: 234–235.

Fung, Y. and Luk, S. 1985a. Determination of ascorbic acid in soft drinks and fruit juices. Part 1. Background correction for direct ultraviolet spectrometry. Analyst. 110: 201–204.

Fung, Y. and Luk, S. 1985b. Determination of ascorbic acid in soft drinks and fruit juices. Part 2. Application of direct ultraviolet spectrometry with background correction. Analyst. 110: 1439–1444.

Jung, M.Y., Choe, E., and Min, D.B. 1991. α-, γ- and δ-tocopherol effects on chlorophyll photosensitized oxidation of soybean oil. J. Food Sci. 56: 807–810, 815.

Jung, M.Y., Kim, S.K., and Kim, S.Y. 1995. Riboflavin-sensitized photooxidation of ascorbic acid; Kinetics and amino acid effects. Food Chem. Submitted.

Jung, M.Y. and Min, D.B. 1991. Effects of quenching mechanisms of carotenoids on the photosensitized oxidation of soybean oil. J. Am. Oil Chem. Soc. 68: 653–658.

Kelly, G.J. and Latzko, E. 1980. Prospect of a specific enzymatic assay for ascorbic acid (Vitamin C). J. Agric. Food Chem. 28: 1320–1321.

Rooney, M.L. 1983. Ascorbic acid as a photooxidation inhibitor. Photochem. Photobiol. 38: 619–621.

Rose, R.C. and Nahrwold, D.L. 1981. Quantitative analysis of ascorbic acid and dehydroascorbic acid by high performance liquid chromatography. Analytical Biochem. 114: 140–145.

Sattar, A., deMann, J.M., and Alexander, J.C. 1977. Light induced degradation of vitamins. Can. Inst. Food Sci. Technol. J. 10: 65–68.

Tsumura, F., Ohsako, Y., Haraguchi, Y. Kumagai, H., Sakurai, H., and Ishii, K., 1993. Rapid enzymatic assay for ascorbic acid in various foods using peroxidase. J. Food Sci. 58: 619–622, 687.

Tono, T. and Fujita, S. 1981. Determination of ascorbic acid in foods by spectrophotometric method based on difference spectra. Agri. Biol. Chem. 45: 2947–2949.

Tono, T. and Fujita, S. 1982. Spectrophotometric determination based on differences spectra of L-ascorbic acid in plant and animal foods. Agric. Biol. Chem. 46: 2953–2959.

Vanderslice, J.T. and Higgs, D.J. 1993. Quantitative determination of ascorbic, dehydroascorbic, isoascorbic, and dehydroascorbic acids by HPLC in foods and other matrices. J. Nutr. Biochem. 4: 184–190.

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Anthocyanin Pigments of Royal Okanogan Huckleberry Juice

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ABSTRACT

Juice processed from Royal Okanogan Huckleberries had an intense purple color, (CIE L*a*b* = L*, 22.66, a*, −0.06, b*, −0.46) and high anthocyanin pigment content, (>5 g/L). The anthocyanins were separated by HPLC and characterized from retention times and UV-visible spectra of purified pigments and their hydrolysis products. The major pigment (>70%) was petunidin 3-(*p*-coumaroyl-rutinoside)-5-glucoside. Eleven additional anthocyanins were identified as petunidin, delphinidin, and malvidin with the same glycosidic substitution pattern as the major pigment, and varying degrees of acylation with *p*-coumaric, ferulic, and caffeic acids. It was concluded from comparative pigment analyses and berry morphology that Royal Okanogan Huckleberries had identical characteristics to the Garden Huckleberry, *Solanaceae melanocerasum* (renamed *S. scabrum*).

Key Words: anthocyanins, huckleberries, *Solanaceae melanocerasum*, *S. scabrum*, petunidin

INTRODUCTION

A PACIFIC NORTHWEST juice processing company supplied us with block-frozen Royal Okanogan Huckleberries for pigment analyses. Royal Okanogan Huckleberries are a new cultivar, being grown in small quantities in the Okanogan valley of north-central Washington state. The berries are highly pigmented, and sauces and preserves with an intense purple color are being processed as a cottage industry and sold in local markets. There was interest to see if the fruit had potential for use in blended juices or juice drinks. We were informed that the Royal Okanogan Huckleberry had parentage from blueberries (*Vaccinium corymbosum* L.), cranberries (*V. macrocarpon* Ait.) and the wild huckleberries native to the Cascade Mountains (blue, *V. ovalifolium* Smith; black, *V. membranaceum* Doug. ex Hook).

The anthocyanin pigment composition of cranberries has been established and confirmed (Macheix et al., 1990). The major pigments are the 3-galactosides and 3-arabinosides of cyanidin and peonidin; the 3-glucosides are present in minor amounts, and the same 3-glycosides of delphinidin are present in trace quantities. The highbush blueberry (*V. corymbosum*) contains the 3-glucosides, galactosides and arabinosides of malvidin, cyanidin, peonidin, delphinidin and petunidin; malvidin-3-glucoside and malvidin-3-galactoside are the major pigments (Macheix et al., 1990). The same anthocyanins have been identified in lowbush blueberry (*V. angustifolium* Ait., Macheix et al., 1990). The anthocyanins of the wild huckleberries native to the Pacific Northwest have not been reported.

Webster's dictionary (1979) defines huckleberries as "any number of related shrubs having dark-blue berries resembling blueberries." Within the family *Ericaceae*, the subfamily *Vacciniaceae* is divided into two tribes (Camp, 1945): the *Gaylussacieae* (true huckleberries) and the *Vaccinieae* (cranberries and blueberries). The *Vaccinium* spp. found in the Pacific Northwest were mislabeled as 'huckleberry' although they are generically *Vaccinium* and not *Gaylussacia* (Stark and Baker, 1992). *Vaccinium membranaceum* (mountain huckleberry, twin-leaved huckleberry), the most extensively harvested *Vaccinium* species in western North America (Luby et al., 1990), is in *Vaccinium* section *Myrtillus* (Vander Kloet, 1988). Included in the *Solanaceae* family is an annual plant known as the Garden Huckleberry (*Solanum guineese* or *S. melanocerasum*). Francis and Harborne (1966) investigated the anthocyanins of the Garden Huckleberry and identified petunidin-3-(*p*-coumaroyl rutinoside)-5-glucoside as the major pigment. Three additional anthocyanins were identified as 3-rutinoside-5-glucosides of petunidin or malvidin, two of which were acylated with cinnamic acids.

Our major objective was to characterize the anthocyanin pigments of the Royal Okanogan Huckleberry and measure their concentration. Juice processing trials were conducted so we could determine the color properties, pigment content and other juice characteristics, including estimated juice yield. The investigation proved to be an interesting case-study in chemotaxonomy.

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MATERIALS & METHODS

Materials

Frozen Royal Okanogan Huckleberries were obtained from Tree Top, Inc., Selah, WA. Purple tomatillos (*Physalis ixocarpa*), eggplants (*Solanum melongena*, var dusky), Highbush blueberries, purple peppers (*Capsicum*, var Chinese Multicolor), and cranberry juice (Welch Foods, Inc.) were purchased from local supermarkets and farms. Garden Huckleberries (*Solanum melanocerasum*) and Mrs. B's non-bitter Garden Huckleberries, also known as Wonderberries (*S. burbankii*) were grown in a home garden from seeds obtained from Peace Seeds, Corvallis, OR. Berries from wild nightshade (*S. nigrum*) were collected in the Corvallis, OR area and identified by pictorial comparison (Underhill, 1989). Blackberry juice was available from an earlier research project (Rommel et al., 1992).

Reagents, solvents and standards

HPLC grade methanol, acetonitrile, glacial acetic acid, acetone, chloroform, reagent grade dichloromethane, hydrochloric acid, potassium chloride, citric acid monohydrate, and 'Dilut-it' sodium hydroxide standard were purchased from J.T. Baker, Phillipsburg, PA. Concentrated phosphoric acid was purchased from EM Science, Gibbstown, NJ. Tris-hydrochloride, caffeic acid, *p*-coumaric acid, and ferulic acid were purchased from Sigma Chemical Corp., St. Louis, MO. Potassium hydroxide, sodium hydroxide, and monobasic potassium phosphate were purchased from Mallinkrodt Chemical Works, St. Louis, MO. Two pectinase enzyme preparations were used in juice processing: Novo Ultra SP (Novo Labs, Danbury, CT) and Rohapect B1-L (Rohm Tech., Malden, MA).

High performance liquid chromatography (HPLC)

Apparatus. Varian Model 5000 liquid chromatograph (Varian Instrument Group, Walnut Creek, CA) fitted with a Hewlett Packard HP 1040A diode array detector (Hewlett Packard, North Hollywood, CA), a Varian refractive index detector, and a Hewlett Packard 9000/9153 computer system.

HPLC columns and gradients. (1) Supelcosil C18 column (Supelco Inc., Bellefonte, PA) 250 mm × 4.6 mm ID fitted with a Biorad ODS-10 guard column (Biorad, Richmond, CA). Solvent A = 15% acetic acid (aqueous), B = 100% acetonitrile. Isocratic at 85% solvent A. Flow rate = 1.5 mL per min. Peaks were monitored at 520 nm and spectra recorded from 250 to 610 nm. (2) Spherisorb ODS-2 column (Alltech Associates, Inc., Deerfield, IL) 250 × 4.6 mm ID fitted with a Biorad ODS-10 guard column. Solvent A = 4% phosphoric acid (aqueous), B = 100% acetonitrile. Initial solvent composition = 12% B with linear

gradient to 16% B in 40 min, followed by linear gradient to 25% B at 45 min. Flow rate = 1.3 mL per min. (3) PLRP-S polymer column (Polymer Labs, Amherst, MA), 5 µm particle size, 250 × 4.6 mm i.d. fitted with a Polymer Labs guard column. Solvents are the same as used with the ODS-2 system (2). Isocratic at 6% B for 10 min, with linear gradient to 20% B at 55 min. Flow rate = 1.5 mL per min. (4) Spherisorb ODS-2 connected in series to a Spherisorb ODS-1 column (both from Alltech Associates Inc., Deerfield, IL) 250 mm × 4.6 mm i.d. Solvent system was as described for HPLC determination of nonvolatile acids in cranberries by Hong and Wrolstad (1986). (5) Biorad Aminex HPX-87C (Biorad Labs, Richmond, CA) 300 × 7.8 mm i.d., fitted with Biorad Carbo C guard column. Solvent and conditions were as described for HPLC determination of red raspberry sugars by Spanos and Wrolstad (1987).

Gas-liquid chromatography (GLC)

Apparatus. Varian Aerograph series 1400 (Varian Instrument Group, Walnut Creek, CA) fitted with an HP-3396 A integrator (Hewlett Packard, North Hollywood, CA). A Supelcoport SP-2340 packed glass column (Supelco, Inc., Bellefonte, PA) 6 ft. × 2 mm i.d. was used. Nitrogen was used as carrier gas at 17 mL/min. The Column was isothermal at 225°C, detector = 262°C, and injector = 240°C.

Juice processing trials

Frozen berries (350 g) were blanched by heating in a microwave oven to 90°C and held for 5 min before cooling to 50°C. A second portion was held at 50°C. Depectinization with Novo Ultra SP pectinase preparation (0.05% v/w) was conducted at 50°C for 3 hr. Depectinization was monitored with an alcohol precipitation test (Rommel et al., 1990). Juice was screened through muslin, filtered successively through Whatman No. 4 filter paper, Polarite polyester fleece material (Fabricland, Corvallis, OR) and through a Buchner funnel with Whatman No. 1 filter paper coated with a 1 cm layer of Hy-flo Super-cell. Trials were done in triplicate.

In a second series of trials, frozen berries (275g) were broken in a Waring Blendor, and blanched by heating to 80°C in a microwave oven and holding for 5 min. Samples not being blanched were held at 50°C. The juice was depectinized with Rohapect B1L (0.1% v/w) at 50°C for 8 hr, followed by 14 hr at 25°C until it tested negative for pectin. Fining with gelatin and bentonite (500 ppm) was as described by Rommel et al. (1990). The filtration procedure was the same as described above; trials were done in duplicate.

Analytical determinations

Titratable acidity was measured as citric acid, according to the AOAC 22.059 glass electrode method, using 0.01 normal NaOH (AOAC, 1984). Juice pH was measured using a Corning 125 pH meter (Corning Glass Works, Medfield, MA) with a Sensorex combination electrode (Stanton, CA.). °Brix of juice samples was determined using a hand held, 0–90° Brix, refractometer (Atago, Japan).

All analytical determinations, including spectral, color, HPLC and GLC analyses were determined in duplicate. The Standard T test was used to determine level of significance.

Sample preparation and HPLC separation of Anthocyanins

Sample preparation was as described by Hong and Wrolstad (1990). Pigments adsorbed on a C-18 Sep-pak cartridge (Water Associates, Milford, MA) were washed with deionized water and eluted with HPLC grade methanol containing 0.01% HCl. After methanol removal, the pigments were dissolved in 4% phosphoric acid and filtered through a 0.45 µm filter. The Supelcosil C-18 system was used for comparative screening of anthocyanins from different materials while the Spherisorb ODS-2-column system (2) was used for most acylated-anthocyanin separations. (The Polymer Labs PLRP-S column system was also effective for separation of acylated anthocyanins.)

Anthocyanins were also isolated from fruit via acetone extraction using the procedure of Wrolstad et al., 1990. The acetone extract was vacuum filtered through a Whatman #1 filter and partitioned with 2.4 volumes of chloroform. Phase separation took place in a separatory funnel over a 5 hr period. Pigments were isolated from the upper, aqueous phase using Sep-pak cartridges as described, and separated via the ODS-2 column system.

Anthocyanidins

Anthocyanidins from juices and purified fractions were analyzed following the procedure of Hong and Wrolstad (1986). Pigments were isolated on a Sep-pak mini-column, rinsed with water, eluted with methanol and dried under vacuum. The isolate was hydrolyzed in 2N HCl at 100°C for 30 min. Modifications included the use of pure methanol to elute pigments from the Sep-pak and the omission of NaCl. Sugars from the hydrolysis procedure were eluted from the Sep-pak cartridge with water and reserved for subsequent analysis. Anthocyanidins were dissolved in 4% phosphoric acid and filtered through a 0.45 µm filter. Pigments were stored at −15°C and analyzed within 12 hr. Anthocyanidins were separated on the Supelcosil C-18 column system.

Alkaline hydrolysis of anthocyanins

The procedure described by Hong and Wrolstad (1990a) was used to saponify the acylating acids and to separate them from the glycosylated anthocyanins. The pigments and acids were washed with deionized water and eluted with HPLC grade methanol before redissolving in 4% phosphoric acid and filtering. Pigments and acids were separated on the Spherisorb ODS-2 column.

Nonvolatile and phenolic acid analysis

Organic acid analysis was carried out following the procedure of Hong and Wrolstad (1986) using ODS columns 1 and 2. Samples and standards were passed through Sep-pak cartridges and filtered through 0.45 µm filters before injecting into the HPLC system. Acylating phenolic acids were separated and identified during the alkaline hydrolysis procedure. Peaks were monitored at 280 nm and authentic samples of caffeic, *p*-coumaric, and ferulic acids were used as standards.

Sugars

Using the method of Blakeney et al. (1983), sugars isolated during the acid hydrolysis procedure were transformed into their corresponding alditol acetates for analysis by gas chromatography. Modifications of the procedure included the use of HCl for sugar hydrolysis and lyophilization to concentrate the sugars in the hydrolysate. Major Sugars of Royal Okanogan Huckleberry Juice were separated by HPLC and identified by retention time according to the methods of Spanos and Wrolstad (1987) using the Varian 5000-LC to deliver the mobile phase. Sugars were monitored with a Varian refractive index detector.

Peak purification

Individual pigments were purified by repeated manual collections from the HPLC separation using the ODS-2 system. Purity of pooled peaks was determined by analysis on the analytical column. Samples were stored at −15°C. Purified pigments were separated from the HPLC mobile phase with a C-18 Sep-pak mini-column.

Anthocyanin content of juice

Total monomeric anthocyanin content was determined according to the method described by Fuleki and Francis (1968). Tannin contribution (%) to the color of the juice was determined using the method described by Somers (1971). Absorptions were measured on a Varian DMS-80 double beam spectrophotometer using quartz cuvettes and appropriate buffer systems as blanks. A 1/100 dilution of single strength juice was necessary to accurately measure absorbance at pH 1 in the measurement of monomeric anthocyanin and a 1/75 dilution for the measurement of polymeric color.

Molar absorption (E) of the major pigment was determined after isolation and identification of the major pigment. The purified pigment was rinsed onto a sep-pak mini column, rinsed three times with deionized water, and eluted with methanol. The solution was evaporated to dryness in a rotary evaporator at 50°C. The pigment was then dried further in a desiccator for 2 hr. The pigment was weighed, recovered by successive rinsings with of 5.0 mL 1% HCl in deionized water, 0.5 mL 1% HCl in methanol, 1.0 mL 1% HCl in deionized water, and made up to 10.0 mL with 1% HCl (aqueous). Further dilution was made to determine the molar absorptivity at 520 and 510 nm.

Absorbances (A) of the solutions were measured on the Varian DMS-80 using 1% HCl (aqueous) as a blank in 1.0 cm quartz cuvettes. The molecular weight of the major pigment, identified as petunidin-3-(*p*-coumaroyl-rutinoside)-5-glucoside, was 934 g/mol.

Table 1—Juice characteristics from processing trials

Processing trial	% Yield	°Brix	pH	TA[a]	Total ACN[b]	Total ACN[c]	% Polymeric Color	Hunter L*	a*	b*
Royal Okanogan huckleberry								22.66	−0.06	−0.46
Rohapect B1-L[d]										
Blanched	69%	5.0	4.4	0.65	5.2	359	33%			
Unblanched	70%	5.9	4.4	0.63	5.2	364	50%			
Novo Ultra SP[e]										
Blanched	60%	5.8	4.4	0.59	5.8	348	32%			
Unblanched	66%	5.4	4.4	0.55	5.0	330	36%			
Solanum melanocerasum	66%	5.0	4.3	0.78	8.1	535		22.70	0.07	−0.58

[a] Titratable acidity as % citric.
[b] Juice total monomeric anthocyanin pigment, g/L.
[c] Fruit total monomeric anthocyanin pigment, mg/100g.
[d] Results are mean of two replications.
[e] Results are mean of three replications.

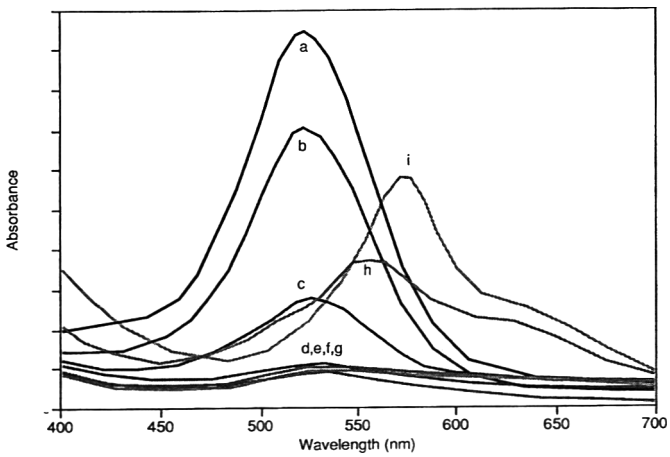

Fig. 1—Visible spectra of Royal Okanogan huckleberry juice diluted 2% with buffer of a, pH 1.17; b, 2.00; c, 3.03; d, 4.05; e, 4.58; f, 5.02; g, 6.03; h, 7.13; and i, 8.01.

Table 2—Instrumental color values for Royal Okanogan huckleberry juice diluted 2% with buffers as related to varying pH

pH	L*	a*	b*	Hue angle[a]	λ_{max} nm
1.17	47.5	58.7	−3.7	356	524
2.00	50.5	56.2	−8.7	351	524
3.03	64.4	34.4	−9.9	344	526
4.05	75.6	10.7	−3.3	343	532
4.58	75.2	8.8	−4.0	336	536
5.02	76.3	6.4	−2.6	338	536
6.03	75.4	3.5	−2.7	322	540
7.13	57.4	11.3	−14.0	309	558
8.01	50.8	−2.5	−15.4	261	578

[a] angle described by the tan $^{-1}$ of a*/b* expressed on a 360° basis where red = 360° = 0; blue = 270°; green = 180°.

Color and spectral analysis

Juice was diluted 1/50 with buffers to determine color and spectral properties at different pH's. Buffers were made by diluting the following compounds in 75 mL water, adjusting the pH with either 1N HCL or 10% NaOH, and bringing the volume to 100 mL with deionized water. pH 1.0 buffer, 1.49 g potassium chloride; pH 2 buffer, 1.28 g monobasic potassium phosphate; pH 3 buffer, 2.38 g citric acid monohydrate; pH 4 buffer, 1.49 g succinic acid; pH 4.5 buffer, 13.6 g sodium acetate; pH 5.0 buffer, 1.45 g succinic acid; pH 6 buffer, 1.27 g succinic acid; pH 7 buffer, 1.33 g monobasic potassium phosphate; pH 8 buffer, 1.64 g tris-hydrochloride. Final pH of the diluted juice solutions were 1.17, 2.00, 3.03, 4.05, 4.58, 5.02, 6.03, 7.13, and 8.01.

CIE L*a*b* values were measured using a Hunter ColorQUEST (Hunter Instruments, Reston, VA) attached to an IBM pc using Hunter Instruments software version 2.5. Transmittance values using standard illuminant C in a 5 mm acrylic cuvette were recorded. UV-visible spectra of the solutions were recorded using an HP 8452 Diode Array Detector Spectrophotometer attached to an HP Vectra scanning computer system (Hewlett Packard, North Hollywood, CA.). Absorbance spectra of the solutions in 1.0 cm pathlength quartz cuvettes were recorded from 400 to 700 nm.

RESULTS & DISCUSSION

Juice characteristics

Table 1 lists characteristics for juices obtained in processing trials. Yields for Royal Okanogan juice ranged from 60–70%, and were significantly higher (Standard T test, $\alpha = 0.05$) with Rohapect B1-L pectinase as compared with Novo Ultra SP. Unblanched fruit gave a higher yield ($\alpha = 0.05$) than blanched fruit. All juices were difficult to filter, even though the negative alcohol precipitation test indicated that depectinization was complete. Considerable foaming occurred during filtration. Juices that had been frozen and subsequently thawed were easier to filter. Fining with Bentonite and gelatin did not improve filtration.

A striking juice characteristic was its pH of 4.4. This was considerably higher than that of cranberries (pH 2.6; Hong and Wrolstad, 1986), blueberries or other *Vaccinium* species. The mean value for titratable acidity of Royal Okanogan juices was 0.61%. Citric acid was identified by HPLC retention as the major non-volatile acid. The single-strength juices were low in Brix (mean = 5.5°). Sugars identified by HPLC were glucose (2.35 g/100 mL), fructose (2.88 g/100 mL) and sucrose (trace). Identification of glucose and sucrose was confirmed by GLC of their alditol acetates. Royal Okanogan Huckleberry juice had an intense purple color. E was determined to be 1.70×10^4. The Anthocyanin pigment concentration in the juice (5.3 g/L) and berries (350 mg/100g) was exceptionally high. Pigment yield was independent of enzyme treatment. Blanching did not have a significant effect on anthocyanin recovery. Polymeric color was significantly higher ($\alpha = 0.10$) in juices that were not blanched.

Color and spectral characteristics

The visible spectra for Royal Okanogan huckleberry juice diluted 2% with buffers of varying pH, were compared (Fig. 1) as well as the CIE L*a*b* measurements and λ_{max} measurements (Table 2). The juice was purple within the pH range 2 to 6, which includes most food applications. At pH 8, it had a blue-green color. Hue angle is conventionally expressed as $\tan^{-1}$ a/b (Francis, 1975). With that convention, there was an abrupt transition from negative to positive with a color change of purple to blue-green. Sapers et al. (1984) circumvented this by normalizing the angles to 360°. This can be visualized by setting (a+, b = 0) to 360°, (also equals 0°) and moving counter clockwise through the Hunter color solid perpendicular to the L axis. Under this scheme pure yellow is represented at 90°, green at 180°, blue at 270°, and red at 360° or 0°. That convention was used for values in Table 2. Hue angle steadily progressed from

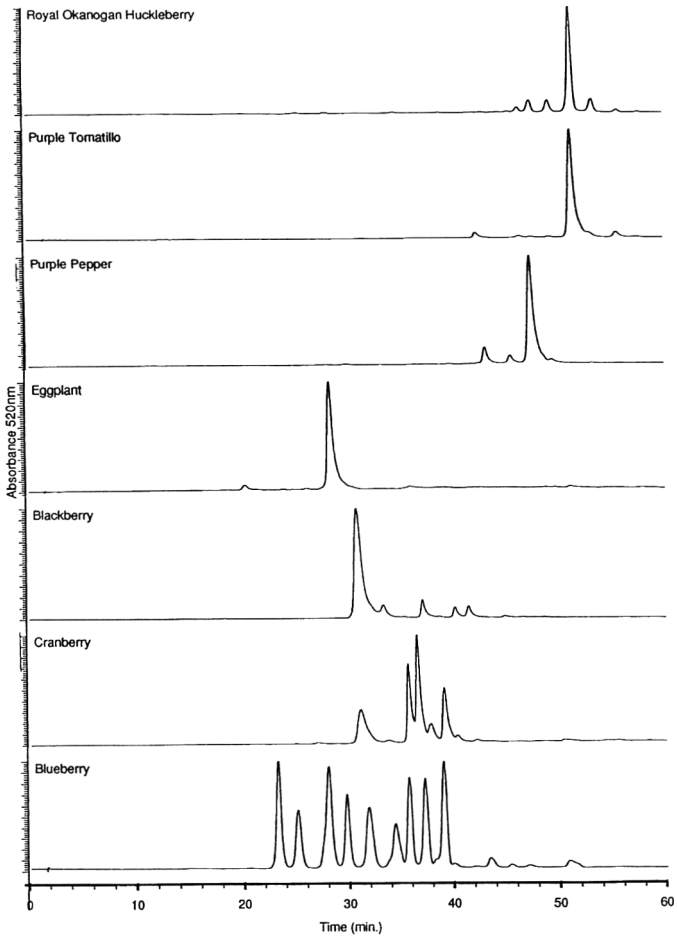

Fig. 2—HPLC anthocyanin pigment profiles of fruits from *Solanaceae, Rosaeae,* and *Ericaceae* families. Column, 4.6 mm i.d. × 250 mm Supelcosil C-18 column; Solvent A = 15% aqueous acetic acid; B = 100% acetonitrile. Isocratic at 85% solvent A; flow rate = 1.5 mL/min; monitored at 520 nm.

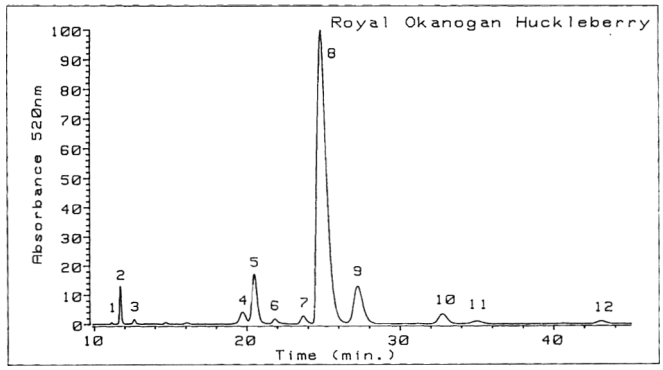

Fig. 3—HPLC chromatogram of Royal Okanogan huckleberry anthocyanins monitored at 520 nm. Column, 4.6 mm i.d. × 250 mm Spherisorb ODS-2 column; Solvent A = 4% phosphoric acid (aqueous); Solvent B = 100% acetonitrile. Initial solvent composition = 12% B with linear gradient to 16% B in 40 min, followed by linear gradient to 25% B at 45 min. Flow rate = 1.3 mL/min.

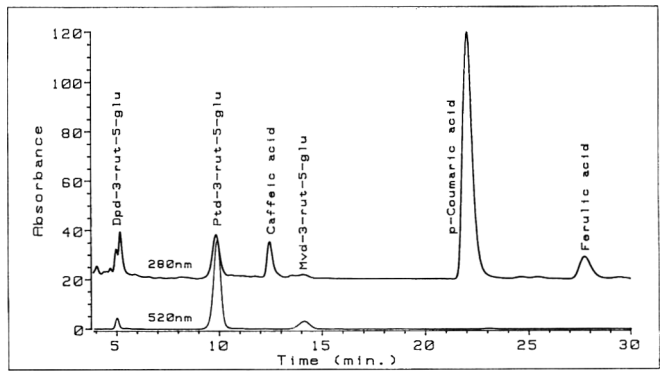

Fig. 4—HPLC chromatogram of deacylated anthocyanin pigments from Royal Okanogan huckleberry, monitored at 520 and 280 nm. (Same HPLC conditions as for Fig. 3).

red-purple to blue-green with increasing pH. A pH change from 1 to 6 resulted in a bathychromic shift of 16 nm (Table 2). While there was considerable change in intensity (Fig. 1), the solutions were purple colored in this range. The single-strength juice at pH 4.4 was extremely dark purple (Table 1).

Characterization of anthocyanin pigments in Royal Okanogan huckleberry

The anthocyanin pigment profiles were compared (Fig. 2) for pigments isolated from Royal Okanogan huckleberries, blueberries, cranberries, blackberries, eggplant, purple peppers and purple tomatillos. The pattern for Royal Okanogan Huckleberry anthocyanins was very different from *Ericaceae* (cranberries, blueberries) and *Rosaceae* families, the longer retention times being more characteristic of *Solanaceae* (tomatillos, peppers). Acylation with hydrophobic cinnamic acids resulted in longer retention times in this reverse-phase separation system. Strong absorption bands at ca. 320 nm confirmed the presence of acylating cinnamic acids in *Solanaceae* and Royal Okanogan huckleberries.

Resolution of Royal Okanogan huckleberry anthocyanins was improved with the ODS-2 column, 12 peaks being resolved (Fig. 3). An absorbance band in the 300–340 nm region indicated that peaks 4 through 12 were acylated with cinnamic acids. The anthocyanins isolated from Royal Okanogan Huckleberries were saponified with KOH to remove acylating acids. This simplified the pigment profile to three anthocyanin peaks which had retention times and spectra identical to peaks 1, 2 and 3 (Fig. 4). By monitoring the hydrolysate at 280 nm, caffeic, *p*-coumaric, and ferulic acids were identified as acylating acids (Figure 4). An unidentified peak eluted 0.25 min before anthocyanin peak 1. We concluded that peaks 4 through 12 were peaks 1, 2 and 3 with different acylation substitution patterns.

Acid hydrolysis of Royal Okanogan huckleberry anthocyanins produced three aglycons. Hydrolysis of blueberry and concord grape anthocyanins generated standards (Hong and Wrolstad, 1990b) for delphinidin (retention time, 11 min), cyanidin (17 min), petunidin (18 min), peonidin (25.5 min), and malvidin (27.5 min); hydrolysis of strawberry anthocyanins produced pelargonidin (24.5 min). The three anthocyanidins in Royal Okanogan huckleberries were identified as delphinidin, petunidin, and malvidin, with petunidin the major aglycon (>80%). The sugars in the hydrolysate were converted to alditol acetates and analyzed by GLC. Glucose and rhamnose were identified, with a molar ratio of glucose:rhamnose, 2:1.

Peaks 4 through 12 were collected and pooled from repetitive HPLC injections for further characterization. Purity of isolates was checked by HPLC analysis. A problem came from contamination from the major pigment (peak 8). Purified anthocyanins were subjected to KOH hydrolysis. The retention time of the deacylated anthocyanin was determined, and the acylated acid identified by HPLC retention time and visible spectrum. The purified peaks were also subjected to acid hydrolysis, and the identity of the aglycon determined by HPLC comparison with standard anthocyanidins. Only peak 8 was collected in sufficient amounts to identify the sugars by GLC of the alditol acetates. It contained glucose and rhamnose in a 2:1 molar ratio (glucose: rhamnose). Another useful property for identification is the ratio

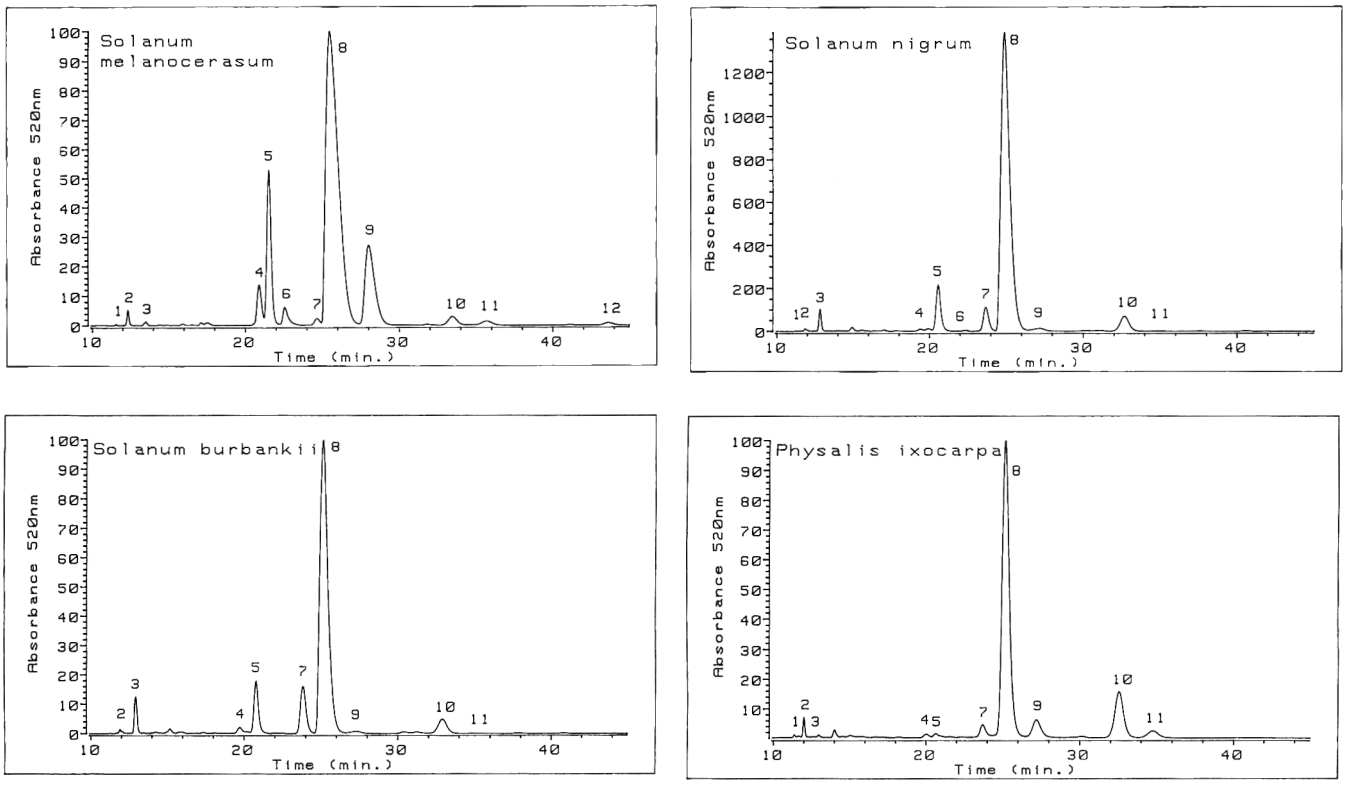

Fig. 5—Comparison of HPLC anthocyanin pigment profiles of *Solanum melanocerasum, S. nigrum, S. burbankii,* and *Physalis ixocarpa,* monitored at 520 nm. (Same HPLC conditions as for Fig. 3).

Table 3—Peak assignments for anthocyanin pigments separated from Royal Okanogan huckleberries

Peak no.	% peak area	DAP[a]	Aglycon[b]	Acyl acid	$A_{440}/A_{\lambda max}$	Peak assignment[b]
1	trace				17	del-3-rut-5-glu
2	1.8				12	pet-3-rut-5-glu
3	0.4				15	mal-3-rut-5-glu
4	2.0	1	del	unknown	13	del-3-acyl-rut-5-glu
5	7.1	1	del	*p*-coumaric	12	del-3-*p*-coumaroyl-rut-5-glu
6	0.8	1	del	ferulic	13	del-3-feruoyl-rut-5-glu
7	1.3		pet	unknown	13	pet-3-acyl-rut-5-glu
8	72.2	2	pet	*p*-coumaric	10	pet-3-*p*-coumaroyl-rut-5-glu
9	9.5	2	pet	ferulic	11	pet-3-feruoyl-rut-5-glu
10	2.5	3	mal	*p*-coumaric	11	mal-3-*p*-coumaroyl-rut-5-glu
11	0.9	3	mal	ferulic	12	mal-3-feruoyl-rut-5-glu
12	0.7	2	pet	*p*-coumaric	13	pet-3-di-*p*-coumaroyl-rut-5-glu

[a] Deacylated peak elutes at same time as peaks 1, 2, or 3.
[b] Abbreviations: rut = rutinoside; glu = glucoside; acyl = unknown acylating acid; pet = petunidin; del = delphinidin; mal = malvidin.

of absorbance at 440 nm to absorbance of the wavelength of maximum absorbance ($A_{440}/A_{\lambda max}$); Harborne (1958) demonstrated that delphinidin, petunidin and malvidin monoglycosides had ratios from 16–19% while the 3–5 diglycosides had ratios from 9–12% (Table 3). The major anthocyanin (peak 8) was identified as petunidin-3-*p*-coumaroyl-rutinoside-5-glucoside. Peak 9 which accounts for over 9% of the total peak area was the same anthocyanin with ferulic acid as the acylating group. Peak 5 (7% of total peak area) was identified as delphinidin-3-*p*-coumaroyl-rutinoside-5-glucoside. For the remaining pigments, insufficient quantities of pure pigments were obtained for conclusive identification. Peak assignments could be made, however, from identification of the deacylated anthocyanin, the acylating acid, and the aglycon. An interesting pattern emerged. The anthocyanins apparently are the 3-rutinoside-5-glucosides of petunidin, delphinidin and malvidin with *p*-coumaric and ferulic acid acylation. Spectral evidence indicated that peak 12 contained two acylating acids.

Species identification of the Royal Okanogan huckleberry

From anthocyanin pigment characterization, it was clear that the information that Royal Okanogan huckleberries were a hybrid of huckleberries native to the Pacific Northwest cascades and other *Vaccinium* (cranberries, blueberries) was incorrect. The major anthocyanin was identical to that identified in the Garden Huckleberry (*S. nigrum* var. *quineese, S. quineese*) by Saito et al. (1965) and Francis and Harborne (1966), respectively. They identified three additional anthocyanins as 3-rutinoside-5-glucosides of petunidin and malvidin, two of which were acylated with cinnamic acids. Morphological characteristics of the Royal Okanogan huckleberry also indicated that it was not related to blueberries or cranberries. When the dark purple Royal Okanogan huckleberry was cut across the equator it resembled a cherry tomato in that it was divided into sections filled with a jelly-like material containing the lenticular, ovoid seeds which were pointed at one end. There were two major sections (locuses) within the fruit where each had a further 'false' sub-division. Pigment was contained throughout the 1 cm diameter fruit. The Royal Okanogan huckleberry is a superior ovary as evidenced by a slight indentation remaining on the bottom of the berry from the attachment of the style, whereas the blueberry is an inferior ovary, retaining an area at the apex where the petals were once attached (Halse, 1991.) In some cases, a small five- or three-lobed calyx attached to a stem re-

mained attached to the frozen berries. Information from the supplier of the fruit indicated that Royal Okanogan huckleberries were present as clusters where several of the berries were attached at the base of their stems in an umbellate fashion. These characteristics are consistent with descriptions for members of the *Solanaceae* (Nee, 1986).

Since we were unable to obtain Garden huckleberry fruit, we obtained seeds from various sources, germinated them indoors in early spring, and planted the seedlings in a home garden once danger of frost had passed. Attempts to germinate the seeds from frozen Royal Okanogan huckleberries were unsuccessful. Fruit from *Solanum burbankii*, *S. melanocerasum* and *Physalis ixocarpa* were harvested in late summer and early fall. Fruit from *S. nigrum* were gathered from wild plants found in the local area. Fruits of *S. melanocerasum* were similar in size (ca. 1 cm) and outward appearances to Royal Okanogan huckleberries. Some informal observations we made during juice processing/extraction with respect to aroma are noted. Both fruits had aroma characteristics reminiscent of potato, with *S. melanocerasum* being slightly more pungent. Royal Okanogan huckleberries had an earthy flavor, with blueberry notes. The flavor of *S. melanocerasum* was more pronounced and had a distinctive 'perfume' in the mouth. These differences could be due to environmental factors or sample storage and do not necessarily indicate that they were different botanically. The berries of *S. nigrum* and *S. burbankii* were smaller (ca. 0.5 cm in diameter) than Royal Okanogan huckleberries, and unlike it, contained most of their pigment in the skin. Purple Tomatillos (*P. ixocarpa*) were 2–3 cm in diameter and were encased in a paper-like calyx.

The anthocyanin pigments from these fruits were isolated (acetone extraction and chloroform partition followed by solid phase extraction utilizing C-18 Sep-Pak cartridges and methanol) and separated by HPLC. Resulting chromatograms (Fig. 5) show retention times and spectra for peaks 8 and 10 were identical for all five fruits. Peak 8, petunidin-3-*p*-coumaroyl-rutinoside-5-glucoside, was the major pigment in all fruits. Note that Mazza and Gao (1994) identified this same pigment as the major pigment in purple-fleshed potatoes (*Solanum tuberosum* L.). Spectral matches were not obtained for all the peaks with matching retention times, possibly because of co-eluting interfering phenolics. The chromatograms of Royal Okanogan huckleberry and *S. melanocerasum* were very similar qualitatively and quantitatively. These two berries had 11 peaks in common with *S. nigrum* and 10 peaks in common with *S. burbankii* and *S. ixocarpa*. Quantitative differences gave pattern variations. The characteristics of the juice processed from *S. melanocerasum* (Table 1) showed that Hunter L*a*b* values, % yield, °Brix and pH were very similar to Royal Okanogan huckleberry. The higher titratable acidity and total anthocyanin pigment content of juice from *S. melanocerasum* could be due to differences in maturity or environmental factors. *S. melanocerasum* has been renamed *S. scabrum* (D'Arcy, 1979; Heiser, 1987; Schilling, 1981). Dr. Richard Halse (1991) of the Department of Botany & Plant Pathology, Oregon State Univ. confirmed that the Royal Okanogan huckleberry was closely related to *S. melanocerasum*, and identified both as *S. scabrum*.

CONCLUSIONS

ON THE BASIS OF ANTHOCYANIN composition and fruit morphology (berry size, pigment distribution, seed location) Royal Okanogan Huckleberry appears to be very closely related to *S. melanocerasum*, if not the same fruit. The intense purple color and high anthocyanin pigment content of Royal Okanogan huckleberry fruit (350 mg/100g) and juice (>5 g/L) make it a potential source for a natural colorant. Over 97% of the anthocyanins are acylated with cinnamic acids which would provide increased stability. Possible applications could be blended juice beverages, or a natural colorant for stamping meats or sausages.

REFERENCES

AOAC. 1984. *Official Methods of Analysis*, 14th ed. Association of Official Analytical Chemists, Washington, D.C.

Blakeney, A.B., Harris, P.J., Henry, R.J., and Stone, B.A. 1983. A simple and rapid preparation of alditol acetates for monosaccharide analysis. Carbohydr. Res. 113: 291–299.

Camp, W.H. 1945. The North American blueberries with notes on other groups of *Vaccinieaceae*. Brittonia 5: 203–275.

D'Arcy, W.G. 1979. The classification of the *Solanaceae*. Ch. 1, in *The Biology and Taxonomy of the Solanaceae*, J.G. Hawkes, R.N. Lester, and A.D. Skelding (Ed.), p. 3–47. Academic Press, New York.

Francis, F.J. 1975. The origin of tan ' a/b. J. Food Sci. 40: 412.

Francis, F.J. and Harborne, J.B. 1966. Anthocyanins of the Garden huckleberry, *Solanum guineese*. J. Food Sci. 31: 524–528.

Fuleki, R. and Francis, F.J. 1968. Qualitative methods for anthocyanins. 2. Determination of total anthocyanin and degradation index for cranberry juice. J. Food Sci. 33: 78–83.

Halse, R. 1991. Personal communication. Dept. of Botany & Plant Pathology, Oregon State Univ., Corvallis, OR.

Harborne, J.B. 1958. Spectral methods of characterizing anthocyanins. Biochem. J. 70: 22–28.

Heiser, C.B. 1987. *The Fascincting World of the Nightshades*. Dover Publications, Mineola, NY.

Hong, V. and Wrolstad, R.E. 1986. Cranberry juice composition. J. Assoc. Off. Anal. Chem. 69: 199–207.

Hong, V. and Wrolstad, R.E. 1990a. Use of HPLC separation/photodiode array detection for characterization of anthocyanins. J. Agr. Food Chem. 38: 708–715.

Hong, V. and Wrolstad, R.E. 1990b. Characterization of anthocyanin containing colorants and fruit juices by HPLC/photodiode array detection. J. Agric. Food Chem. 38: 698–708.

Luby, J.J., Ballington, J.R., Draper, A.D., Pliszka, K., and Austin, M.E. 1990. Blueberries and cranberries, in *Genetic Resources of Temperate Fruit and Nut Crops*, J.N. Moore and J.R. Ballington (Ed). Acta Horticultura 290. International Society for Horticultural Science, Wageningen, The Netherlands.

Macheix, J., Fleuriet, A. and Billot, J. 1990. *Fruit Phenolics*. CRC Press, Inc., Boca Raton, FL.

Mazza, G. and Gao, L. 1994. Acylated anthocyanins in purple hulls of sunflower seeds and purple-fleshed potatoes. *Proceedings of the First International Symposium on Natural Colorants*, 11/7–10/93, Amherst, MA. P.C. Hereld (Ed.). S.I.C. Publishing Co., Hamden, CT.

Nee, M. 1986. Placentation patterns in *Solanaceae*, in *Solanaceae: Biology and Systematics*. W.G. D'Arcy, (Ed.), p. 169–175. Columbia University Press, New York.

Rommel, A., Heatherbell, D.A., and Wrolstad, R.E. 1990. Red raspberry juice and wine: Effect of processing and storage on anthocyanin pigment composition, color and appearance. J. Food Sci. 55: 1011–1017.

Rommel, A., Wrolstad, R.E., and Heatherbell, D.A. 1992. Blackberry juice and wine: Processing and storage effects on anthocyanin composition, color and appearance. J. Food Sci. 57: 385–391, 410.

Saito, N., Kenzo, A., Honda, T., Timberlake, C.F., Bridle, P. 1985. Acylated delphinidin glucosides and flavonols from *Clitoria teratea*. Phytochemistry 24: 1583–1586.

Saito, N., Hotta, R., Imai, K., and Hayashi, K. 1965. On petanin isolated from the berries of *Solanum nigrum*, var. *guineense*. Proc. Japan. Academy 41: 593–598.

Sapers, G.M., Taffer, I., and Ross, E.R. 1981. Functional properties of a food colorant prepared from red cabbage. J. Food Sci. 46: 105–109.

Sapers, G.M., Burgher, A.M., Phillips, J.G., and Jones, S.B. 1984. Color and composition of highbush blueberry cultivars. J. Amer. Soc. Hort. Sci. 109: 105–111.

Schilling, E.E. 1981. Systematics of *Solanum* section *Solanum* (*Solanaceae*) in North America. Syst. Bot. 6: 172–185.

Somers, T.C. 1971. The polymeric nature of wine pigments. Phytochemistry 10: 2175–2186.

Spanos, G.A. and Wrolstad, R.E. 1987. Anthocyanin pigment, nonvolatile acid, and sugar composition of red raspberry juice. J. Assoc. Off. Anal. Chem. 70: 1036–1046.

Stark, N. and Baker, S. 1992. *The Ecology and Culture of Montana Huckleberries: A Guide for Growers and Researchers*. Montana Forest & Conservation Experiment Station Publication 52, Bozeman, MT.

Underhill, J.E. 1989. *Northwestern Wild Berries*, 2nd ed. Hancock House Publishers, Ltd., Blain, WA.

Vander Kloet, S.P. 1988. The Genus *Vaccinium* in North America. Agriculture Canada Publication No. 1828, Ottawa, Canada.

Webster by McKechnie, J.L. 1979. *Webster's Unabridged Dictionary*, 2nd ed., Simon and Schuster, New York.

Wrolstad, R.E., Skrede, G., Lea, P., and Enersen, G. 1990. Influence of sugar on anthocyanin pigment stability in frozen strawberries. J. Food Sci. 55: 1064–1065, 1072.

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Ultrasonication, Lyophilization, Freezing and Storage Effects on Fat Loss during Mechanical Infusion of Expressed Human Milk

J. DHAR, A.G.F. DAVIDSON, F.E. MARTINEZ, S. BARR, I.D. DESAI, and S. NAKAI

ABSTRACT

Ultrasonic homogenization was extended to situations where expressed human milk needs to be stored before being administered. We investigated whether the effect of ultrasonication would persist during storage in the frozen or lyophilized form. Recovery of fat was higher in ultrasonicated and frozen milk (stored for both 1 and 4 mo), than in milk stored following ultrasonication and lyophilization. The low fat recovery from stored lyophilized milk was increased by ultrasonicating the milk after storage and reconstitution (instead of prior to storage). Protein recovery was virtually complete with both methods.

Key Words: human milk, ultrasonication, lyophilization, fat separation

INTRODUCTION

WHEN ILLNESS OR PREMATURITY NECESSITATES that an infant be fed human milk via gavage or ostomy tube, significant losses of milk fat occur due to adherence to the feeding tube (Greer et al., 1984; Stocks et al., 1985; Martinez et al., 1987). Cumulative losses of up to 47.4% of the fat were reported by Martinez et al. (1987) when expressed breast milk was infused at a slow rate, using a mechanical pump. These results raised several concerns relevant to the feeding of human milk to low-birth-weight infants by nasogastric infusion. A notable proportion of fat that separates from expressed human milk (EHM) during tube feeding becomes unavailable to the baby. In mature human milk, where fat accounts for 50% of total calories (Garza et al., 1987), 15% to 20% of the total caloric value of milk may be lost (unavailable) in such delivery systems. Greer et al. (1984) found that if tubing with residual milk was discarded after the syringe or burette was empty, even more fat was wasted and became unavailable (up to 50% of total fat and 27.5% of intended total calories). Also the extent of losses of fat associated substances such as fat soluble vitamins and trace minerals is unknown. Altered protein/calorie ratio of human milk feedings by the loss of fat with continuous pump infusions are also possible (Martinez et al., 1987). An important clinical implication, therefore, would be that these nutrient losses may tend to invalidate studies comparing the influence of feeding human milk or formulae on the growth of low birth weight babies. Similarly, nutrient losses may be a potential cause for lower weight gain shown by babies fed continuously by the naso-jejunal route compared with those fed with intermittent boluses (Whitfield, 1982).

Martinez et al. (1987) demonstrated that these losses of fat during tube feeding could be prevented by homogenizing human milk using ultrasonication to form a stable suspension with small, uniformly sized fat globules. Although cow's milk is routinely homogenized commercially, using high pressure to rupture the fat globules, this method is not suitable for processing human milk, as available equipment requires large volumes and is also very costly. The effectiveness of ultrasonic homogenization in preventing fat loss during tube feeding has been demonstrated by ultrasonicating freshly expressed human milk just before tube feeding (Martinez et al., 1987). However, in clinical practice, the milk is often pasteurized and stored prior to feeding the infants. Our objective was to determine the effect of ultrasonic homogenization and storage, (either frozen or lyophilized) on the recovery of fat and protein in breast milk during simulated infusions.

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MATERIALS & METHODS

Milk collection

Expressed human milk (EHM) samples (~65 mL each) were obtained from the B.C. Children's Hospital, Lactation Support (Breast Milk) Ser-

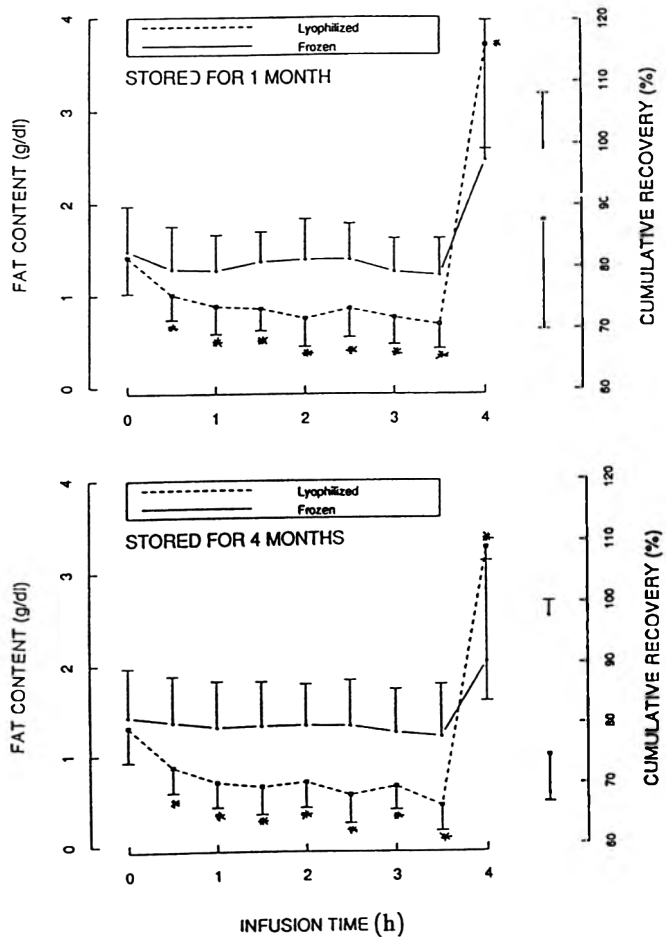

Fig. 1—Periodical and cumulative changes in fat content of ultrasonicated-frozen and ultrasonicated-lyophilized milk infused at a slow rate (10 mL/hr). Each point represents Mean ± SD of eight samples. * = Differences from zero time concentration are significant.

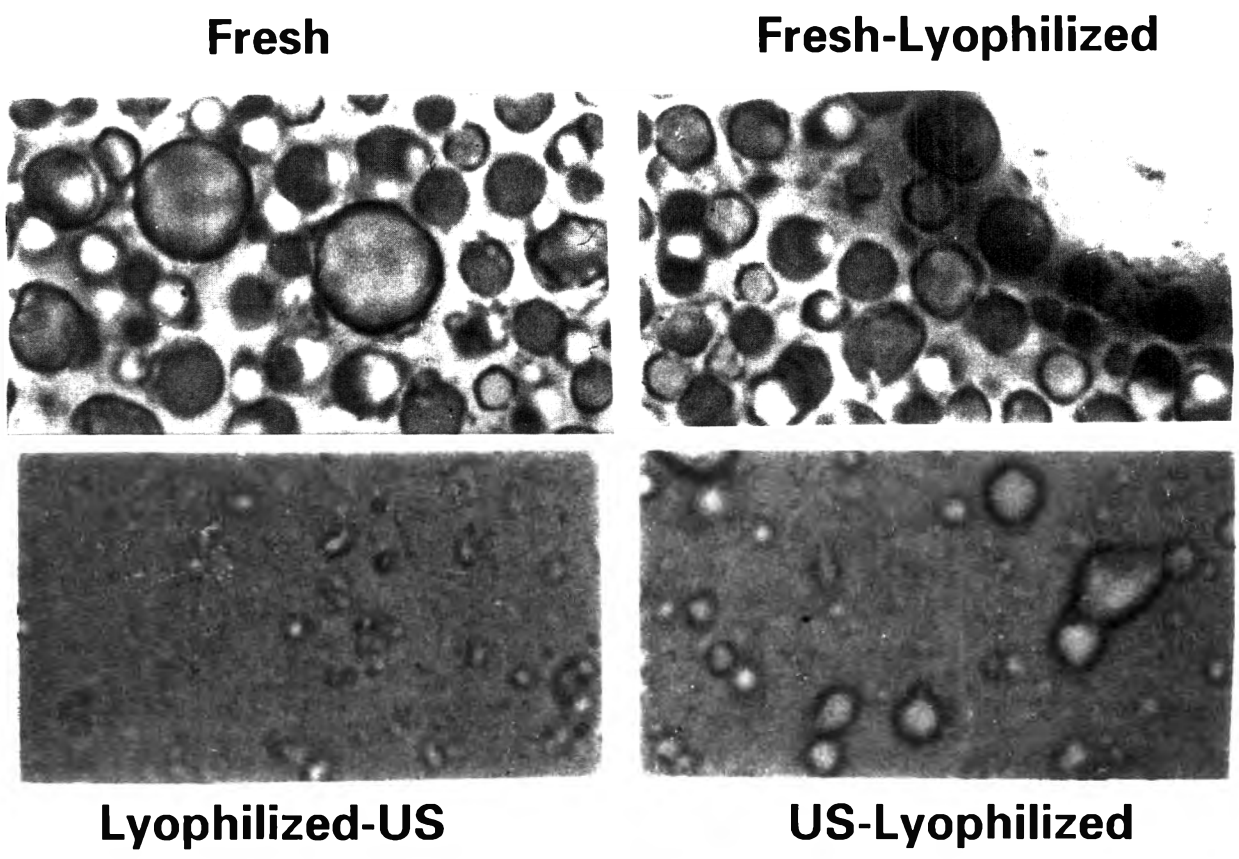

Fig. 2—Photomicrographs showing number of fat globules in fresh and processed human milk. Fresh (80/mm^2), lyophilized (64/mm^2), ultrasonicated-lyophilized (106/mm^2) and lyophilized-ultrasonicated (150/mm^2). All four milk samples photographed at same magnification.

vice in Vancouver, Canada. All samples were predominantly fore milk (i.e., initial ~65 mL) expressed by mothers lactating between 3–6 mo postpartum under the supervision of the breast milk service coordinator. All samples were collected between 2 and 4 pm and pasteurized in the Oxford Human Milk Pasteurizer at B.C. Children's Hospital, Vancouver, for 30 min at 63°C and followed by a rapid cooling cycle. Eight samples were used for the first part of the investigation i.e., to study the effect of freezing and lyophilization on fat and protein loss during mechanical infusion of ultrasonicated milk. Each milk sample was a pool of four EHM samples of around 65 mL collected from four different mothers on the same or next day. Five samples were used for the second part of the investigation to study whether ultrasonication of milk to prevent fat loss should be done before or after lyophilization. Each sample was a pool of two EHM samples of around 65 mL obtained from two different mothers on the same or next day.

Ultrasonic treatment

The conditions for ultrasonic homogenization were as described by Martinez et al. (1987). About 65 mL of milk was ultrasonicated at a relative intensity of 5 (on a scale of 1 to 10) for 5 min. The apparatus used was a Tekmar Sonic Disruptor, Model TSD-P 250, 500 Watts, 20 Hz (Tekmar Co., Cincinnati, OH).

Effect of freezing and lyophilization on ultrasonicated milk

Eight EHM samples were collected, pasteurized and ultrasonicated. Each sample was divided into four parts. Two were stored frozen at −20°C in airtight plastic containers for 1 and 4 mo. The remaining two were lyophilized using a lab-conco Model 75018-18 L Freeze drier (Lab Conco Corp., Kansas City, Mo). Milk samples to be lyophilized were placed in containers with a large surface area to facilitate drying; they were then frozen, covered with parafilm with a few small holes in it and then placed in the freeze drier for ≈3 days. After the samples were dried, they were removed, flushed with nitrogen gas, and sealed tightly for storage for either 1 or 4 mo.

When samples were removed from storage following 1 or 4 mo, frozen samples were thawed around 22°C and lyophilized samples were reconstituted. These samples were then infused, using a laboratory model simulating tube feeding in clinical practice, as described by Martinez et al. (1987). Infusions were made either slowly, using a mechanical syringe pump at 10 mL/hr or rapidly, using gravity flow from an open syringe at 40 mL/hr. Aliquots of milk were obtained both before and during infusions. During rapid infusion, aliquots were collected for 6 min periods, for a total of 30 min, whereas during slow infusion, aliquots were collected over 30 min periods for 4 hr. All aliquots were stored at −20°C prior to analysis for fat and protein.

Ultrasonication before or after lyophilized storage

Five EHM samples were collected, pasteurized and divided into two parts. One part was ultrasonicated, lyophilized and stored for 1 mo, after which it was reconstituted, re-ultrasonicated and infused slowly. The other part was lyophilized and stored for 1 mo after which it was reconstituted, ultrasonicated and then infused slowly, as described. Fat recovery was assessed in infused samples.

Analysis

Samples were analyzed for fat and protein by the method of Nakai and Chi (1970). Acetic acid (97%) was used for dissolving both proteins and fat to obtain a clear and colorless solution from whole milk. Protein was measured by reading absorbance at 280 nm. Fat content was determined from turbidity at 400 nm after addition of urea-imidazole solution.

Calculations and statistics

Total fat recovery was obtained by adding the amount of fat in the aliquots collected after infusion. The cumulative percentage of fat or protein recovered was determined from the fat content in the milk before infusion (concentration × total volume) and the total amount recovered after infusion. Statistical analysis of the data (difference between each time point and zero time) was done using t tests.

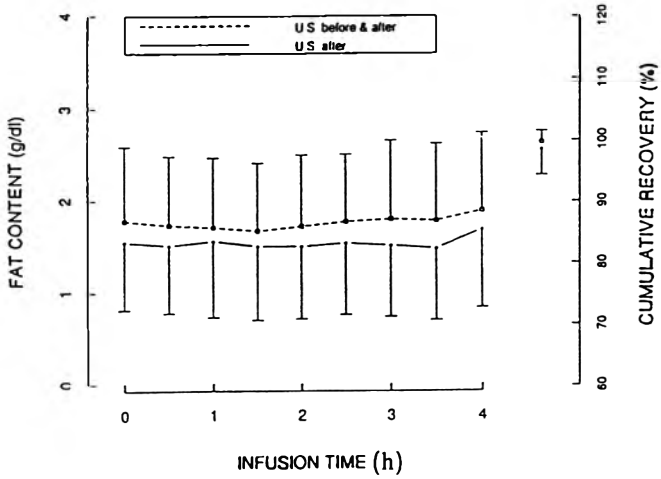

Fig. 3—Changes in fat content of milk ultrasonicated after lyophilization and storage and both before and after lyophilization and storage and infused at a slow rate (10 mL/hr) using mechanical pump. Each point represents Mean ± SD of five samples. U S = Ultrasonicated.

RESULTS & DISCUSSION

Fat recovery after storage of frozen and lyophilized ultrasonicated milk

Fat content and total fat recovery from ultrasonicated milk when mechanically infused slowly (10 mL/hr) after frozen or lyophilized storage for 1 to 4 mo were compared (Fig. 1). Recovery of fat from slow infusion of ultrasonicated and frozen milk was greater ($p < 0.01$) compared to recovery from ultrasonicated and lyophilized milk, whether stored for 1 or 4 mo. The cumulative (over 4 hr) recoveries of fat in ultrasonicated milk frozen for 1 and 4 mo were 99.2% and 97.7%, similar to recovery from freshly sonicated milk. In these samples the concentration of fat was relatively constant over the entire infusion period although some increase was noted in the last aliquot. These results, therefore, suggest that storage of ultrasonicated milk for up to 4 mo does not reverse the homogenizing effects of ultrasonication.

On the other hand, for ultrasonicated milk which had been lyophilized and stored for 1 or 4 mo (Fig. 1), fat content fell significantly in the first 30 min of infusion and remained low. During the final 30 min of infusion, a sharp rise in fat concentration was observed. This pattern was also noticed in milk infused without any ultrasonication (Martinez et al., 1987). This large fat bolus delivered at the end is of great concern. Premature infants are known to absorb fat inefficiently and such boluses of fat may lead to feeding intolerance and also delay advancement of enteral feedings in the infants (Alemi et al., 1981). Cumulative recoveries from samples stored for 1 and 4 mo were 87.5% and 74.1%. Although this was higher than the 52.6% recovery of fat reported by Martinez et al. (1987) with slow infusions of nonhomogenized milk, it is not as high as freshly sonicated milk or sonicated and frozen milk. These results clearly suggest that the advantages of ultrasonicating milk are reduced with lyophilized storage. As demonstrated (Fig. 2, showing number of fat globules in ultrasonicated-lyophilized milk) the process of lyophilization coalesces fat globules in milk (i.e., reversing homogenization effects and thereby leading to low fat recovery).

In the rapidly infused samples almost 100% of the fat in the initial aliquot was recovered after 1 and 4 mo storage in the frozen state (infusion rate 40 mL/hr). For ultrasonicated and lyophilized milk, 93.54% and 84.28% of initial fat content was recovered in the milk stored for 1 and 4 mo. The difference between the frozen and lyophilized samples was significant at both 1 and 4 mo ($p < 0.05$).

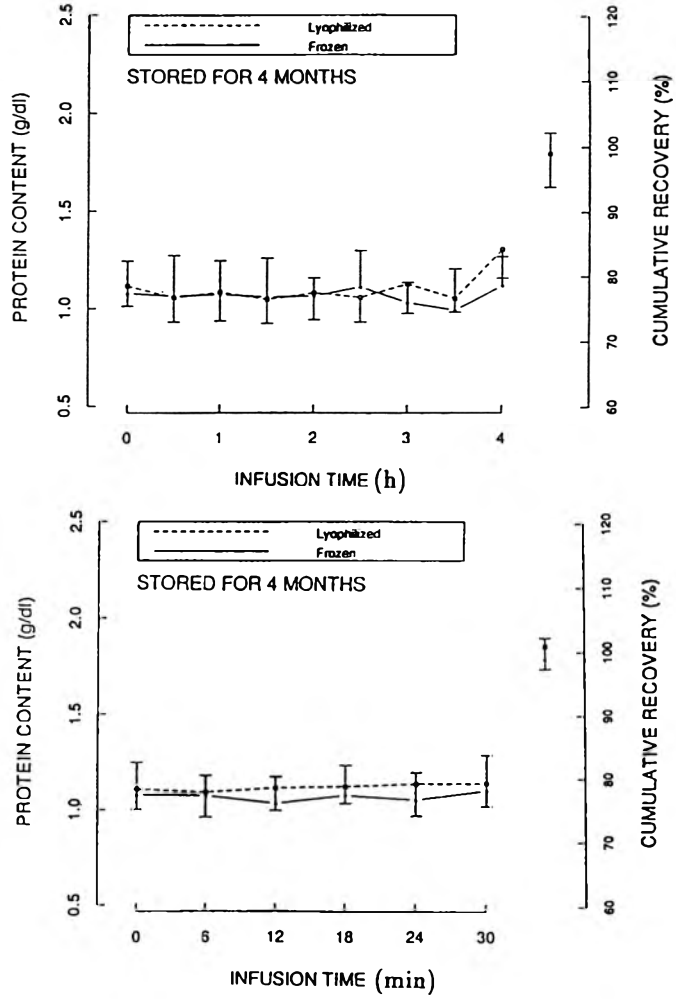

Fig. 4—Periodical and cumulative changes in protein content of ultrasonicated-frozen and ultrasonicated-lyophilized milk infused at a slow rate (10 mL/hr) and at a rapid rate (40 mL/hr). Each point represents Mean ± SD of eight samples.

Fat recovery after storage and subsequent ultrasonication of lyophilized milk

Recovery of fat in infusates which had been ultrasonicated after lyophilization, storage and reconstitution (Fig. 3) was compared. Milk samples with and without ultrasonication were stored for 1 mo in the lyophilized state. The recovery of fat appeared similar and consistent throughout the infusion time of 4 hr for milk ultrasonicated once (after lyophilization and storage), as well as for milk ultrasonicated twice, (i.e., before and after lyophilization and storage). In both groups, recovery of fat was also similar, 98.5% and 99.6%, respectively. This suggested that ultrasonicating milk after lyophilized storage prevented fat loss during infusion. These effects are shown in photomicrographs (Fig. 2) which illustrate fresh, fresh lyophilized, ultrasonicated-lyophilized and lyophilized-ultrasonicated milks. Milk that was ultrasonicated after lyophilization had more numerous, more uniform and smaller fat globules/mm^2 compared to milk that was ultrasonicated before lyophilization. These results, therefore, indicate the need of ultrasonication to be adapted for stable recovery of fat during tube feeding frozen stored milk, and for disrupting the aggregation of fat globules which seems to occur during lyophilized storage.

Protein recovery after storage of frozen and lyophilized milk

Periodical and cumulative changes were compared (Fig. 4) in protein content of ultrasonicated frozen and ultrasonicated lyo-

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UHT-Sterilized Peanut Beverages: Changes in Physicochemical Properties during Storage

ISMAIL Y.S. RUSTOM, M.H. LÓPEZ-LEIVA and BABOO M. NAIR

ABSTRACT

Strawberry-flavored and chocolate-flavored peanut beverages were produced in a pilot plant. UHT sterilized at 137°C for 4 sec and 20 sec, aseptically filled and stored at 5, 20 or 35°C. Microbiological and physicochemical properties of the beverages were periodically assessed for up to 6 mos. No microbial growth was observed. The pH decreased while homogenization and sedimentation indices increased with time in all beverages at all temperatures. Color lightness decreased during the first 6 wk and remained constant afterwards. Viscosity of strawberry-flavored beverage was constant whereas chocolate-flavored beverages gelled after 19 wk at all temperatures. Proteolysis was <6% in gelled beverages.

Key Words: peanut beverage, storage effects, proteolysis, UHT, gelation

INTRODUCTION

AN ALTERNATIVE to conventional in-container food sterilization is ultra high temperature (UHT) processing. The product is heated at 135–150°C for a few seconds followed by rapid cooling (Burton, 1988). UHT sterilization and aseptic packaging are widely used in processing dairy products, fruit juices, soups, custards, catering mixes, and liquid foods containing particulate solids (Burton, 1988; Zadow, 1993) and soy milk (Narayanan et al., 1993).

Physicochemical changes occurred in UHT stored milk (Harwalkar and Vreeman, 1978; Mogensen and Poulsen, 1980; Andrews, 1986; Renner, 1988; Reddy et al., 1991). The changes were pH drop, increase in proteolysis, age gelation, oxidative rancidity, fat separation, sediment formation and development of Maillard reaction products. Such changes result in deteriorative effects on product quality, and hence became more limiting factors for shelf-life than microbial spoilage.

We investigated and optimised the extraction of peanut solids with water to develop an extract that could be utilised as a nutritious beverage (Rustom et al., 1991a,b; 1993). For the current study strawberry-flavored and chocolate-flavored peanut beverages were produced in a pilot plant. They were UHT sterilized and aseptically packed in Tetra Briks®. Our objective was to study the effects of storage temperature and time on some physicochemical properties of the products.

MATERIALS & METHODS

Materials

Peanuts, *Arachis hypogaea L.*, imported from Thailand, were purchased from the local market (Bangkok Trading, Malmö, Sweden). Crude papain (P 3375, activity: 1.5–3.5 units/mg solid, lactose-free) was from Sigma Chemical Co., St. Louis, MO, USA. Recodan EMS® (mono-and-diglycerides + glycerol monostearates + soya lecithin) and Recodan CM® (mono-and-diglycerides + glycerol monostearates + guar gum + carrageenan) were from Grindsted, Brabrand, Denmark. Red colorant (Hushållsfärg) and vanilla aroma (Hushålsarom) were from Ekströms Konsumentkontakt, Örebro, Sweden. Strawberry flavor was from Saturnus AB, Malmö, Sweden. Rom-Cacao flavor was from AB Einar Willumsen, Malmö, Sweden. Sugar and cacao powder were purchased from the local market in Lund, Sweden.

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Beverage processing

Peanut extract was prepared at the Dept. of Food Engineering, Chemical Centre, Lund University, Lund, Sweden, one day before being processed to a final beverage in a pilot plant located at Tetra-Laval Food Company, Lund, Sweden. The extract, after addition of sugar, emulsifier, flavor and colorant, was processed into three different beverages, 100L each, coded S04, C04 and C20. Beverage codes, formulations, UHT treatment and storage conditions are listed (Table 1). Three batches, 120L each, of the extract were prepared as outlined (Fig. 1). The preparation, grinding, extraction, filtration and clarification were carried out batchwise, whereas, mixing, homogenization, sterilization and aseptic packaging were performed in a continuous process. Detailed descriptions of the operations are given below:

Preparation

Peanut kernels were heated at 80°C in an electric oven with air circulation (IFÖ Kampri, type T 2008, Kampri Storkök, Sweden) for 20 min followed by mechanical abrasion of kernels between 2 rough metal plates (by hand). Peanut red skins (testa) were thereafter manually separated from the kernels.

Grinding and extraction

The clean testa-free kernels were ground twice in a meat mincer (Electrolux, type 1987/R20, Electrolux, Denmark) to a paste. The paste was stored at 5°C for later use. Two extraction steps were carried out in a jacketed-type stainless steel tank heated with water. The tank was equipped with a pneumatic temperature controller and with two motor-driven propellers. In the first-stage extraction, 90 L of tap water was heated in the tank to 50°C followed by adjustment of pH to 8.0 with 1N NaOH. Papain powder (52.5g) was thereafter dissolved in the mixture. The peanut paste (15 kg) was added and the mixture was continuously stirred for 30 min, while keeping the temperature constant at 50°C. In the second-stage extraction, remaining peanut solids (residue) from the first stage were extracted with 30 L water for 30 min at 50°C and pH 8.0.

Filtration, clarification, and mixing

The extraction mixture was filtered through a nylon filter of uniformly shaped identical pores (160 × 120 μm). Larger particles were removed from the extract using a disc bowl separator (Alfa-Laval Co., type 29AE/1963, Lund, Sweden) operated at 4000 rpm. The clarified extract was stored at 5°C overnight (15 hr). The extract was heated to 65°C in a steam-jacketed tank, agitated with an impeller (Bredo mixture, Paul Muller Co., Germany). Sugar, emulsifier, flavor and colorant were added and mixed thoroughly for 20 min at 65°C.

Homogenization and sterilization

An indirect UHT equipment (Sterilab®, Alfa-Laval, Lund, Sweden) with upstream homogenization was used. The beverage mix was transferred from the Bredo mixer to the UHT feed tank, pumped at 55 ± 2°C to a pre-heater where its temperature was increased to 73 ± 1°C. The pre-heated mix was homogenized at 72°C in a two-valve homogenizer (Alfa-Laval, type SHL 05A, Lund, Sweden) at 200 kg/cm^2 in the first valve and 50 kg/cm^2 in the second. The beverage temperature was thereafter raised to 138°C in a heat exchanger and maintained constant at 137°C in a holding tube for 4 sec and for 20 sec. Immediately after

Table 1—Peanut beverage formulations, UHT treatment and storage conditions

Beverage	Composition (g/100 g beverage)						UHT treatment	Storage temp (°C)	Code
	Extract	Sugar	Emulsifier	Flavor	Colorant	Cacao powder			
Strawberry-flavored (S04)	91.3	8.0	0.3[a]	0.3[b]	0.1[c]	0.0	137°C, 4 s	5	S0405
								20	S0420
								35	S0435
Chocolate-flavored (C04)	90.3	8.3	0.4[d]	0.4[e], 0.10[f]	0.00	0.5	137°C, 4 s	5	C0405
								20	C0420
								35	C0435
Chocolate-flavored (C20)	90.3	8.3	0.4[d]	0.4[e], 0.10[f]	0.00	0.5	137°C, 20 s	5	C2005
								20	C2020
								35	C2035

[a] Recodan EMS®. [b] Strawberry. [c] Red. [d] Recodan CM®. [e] Rom-Cacao. [f] Vanilla.

the holding tube, the beverage was cooled to 78 ± 2°C in a pre-cooler, and to 20 ± 2°C in a final cooler.

Aseptic packaging

The sterilized product was collected in an aseptic tank and thereafter pumped to a filling machine (Tetra Pak, type AB3-250, Tetra Pak, Lund, Sweden). The product was aseptically filled in 250 mL Tetra Brik® cartons (ABS Doplex, 175 g/m²). The Briks were made of composite layers of: Low density polyethylene/paper board/aluminium foil/low density polyethylene.

Storage experiment

Eighty Briks of each beverage were stored at each temperature of 5, 20, or 35°C for 6 mos. Physicochemical properties were measured at intervals of 0, 2, 6, 8, 11, 15, 17, 19, 21 and 24 wk. Microbiological analysis was conducted monthly.

Microbiological assay

The standard International Dairy Federation (IDF) procedures were followed to determine total aerobic plate counts (IDF, 1987) and counts for yeasts and molds (IDF, 1990).

Physicochemical properties

pH. The pH was measured at 20 ± 2°C with an electronic pH meter (PHM62, Radiometer, Copenhagen, Denmark).

Extent of proteolysis. Total nitrogen (TN) in the beverage was fractionated into non-protein nitrogen (NPN) and protein nitrogen (isoelectric precipitate). Extent of proteolysis was calculated from the difference between relative amounts of NPN to TN, expressed as a percentage, at 0 and 24 wk storage (Renner, 1988). NPN was determined according to a modification of the procedure described by Harwalkar and Vreeman (1978). The beverage was diluted (1:1, V/V) with distilled water, and skimmed by centrifugation (2500 × *g*, 20°C, 16 min). The pH of skimmed beverage was adjusted to 4.6 with 1N HCl. The acidified beverage was warmed to 50°C for 3 min, cooled to room temperature and centrifuged (2500 × g, 20°C, 30 min). Nitrogen in the original beverage (TN) and in the supernatant (NPN) was determined with a Kjeltic Auto 1030 Analyzer (Tecator AB, Höganäs, Sweden).

Viscosity. Viscosity was determined at 20°C using a falling ball viscometer (Höppler Viskometer, model CH 2015, Medingen, Germany). The viscosity was calculated as:

$$\eta = t\,(W_b - W_s)\,C_b$$

Where η = viscosity (mPas), t = falling time of the ball (s), W_b = specific weight of the ball (g/mL), W_s = specific weight of the sample (g/mL), C_b = ball constant.

Homogenization index. This was determined according to a procedure modified from a method described by the Swedish Dairy Association, SMR, (Anonymous, 1970) as follows: Two days before analysis, two Briks of beverage were transferred from storage cabinets and kept standing upright undisturbed at 20°C. The Briks were thereafter opened, being careful not to disturb them, and 50 mL (A) was pippeted from the upper layer—10% of the Brik total height. Another 50 mL (B) were pippeted from the 90% lower layer. The fat content in A and B was determined using Gerber method (Anonymous, 1970). A and B were each thoroughly mixed after collection and before fat analysis. The homogenization index was calculated as:

$$HI = 100\,(a - b)/a$$

Where HI = homogenization index (%), a = fat content in A, b = fat content in B.

Sedimentation index. A procedure modified from a method described by Alfa-Laval company (Anonymous, 1983) was used to measure sedimentation index. The beverage was thoroughly mixed, and 12 mL were transferred to a flat-bottomed glass tube (13 cm height, 2 cm diameter). Height of the tube was calibrated with known water volumes. A calibration curve was thus obtained in which 0.4–2.5 cm height corresponded to 0.05–2.2 mL volume. The samples were centrifuged (Funk-Gerber GmbH, Berlin, Germany) at 20 ± 2°C, 1100 ± 50 rpm for 20 min. Immediately after centrifugation, the sediment height was measured and the corresponding sediment volume was obtained from the calibration curve. Sedimentation index was expressed as mL/12 mL.

Color. A tristimulus colorimeter (Dr. Lange Micro Color, Dr. Bruno Lange GmbH, Berlin, Germany) was used to measure color. The CIE-LAB color solid with L*a*b* coordinates was adopted (L* = 0; black, L* = 100; white), (a* = −80; green, a* = +100; red), (b* = −70; blue, b* = +70; yellow). The colorimeter was calibrated against black (open air) and white (Dr. Lange standards, no. LZM076) standards. Hue and chroma were calculated as:

$$\text{hue} = \tan^{-1}(b^*/a^*)$$

$$\text{chroma} = \sqrt{a^{*2} + b^{*2}}$$

Color measurements were performed on 80 mL beverage in a 100 mL plastic cup completely wrapped with black light-absorbing adhesive tape to eliminate extraneous light.

Statistical analysis

Statistical analyses were carried out using STATGRAPHICS software (STATGRAPHICS, 1991). Linear regression models for sedimentation index on storage time were fitted using Simple Regression module with 95% confidence level. Multifactor Analysis of Variance (ANOVA) module was used to perform 2-way ANOVA with storage temperature and time as factors. Multivariate Methods module was used to calculate Pearson product-moment correlation coefficients between changes in physicochemical properties during storage. Paired t-test was conducted on extent of proteolysis at 0 and 24 wk storage.

RESULTS & DISCUSSION

Microbiological quality

No microbial growth was observed in any of the beverages stored at 5, 20 or 35°C for 24 wk. Therefore, microbiologically, UHT treatments at (137°C, 4 sec) or at (137°C, 20 sec) were equally effective in extending keeping quality beyond 6 mo.

Physicochemical properties

pH. Effects of storage temperature and time on pH of the beverages were compared (Fig. 2). During mixing, pH was adjusted to 8.0. However, after UHT treatment pH of the strawberry-flavored and chocolate-flavored beverages decreased to 7.84 and 7.73 respectively. Probably the heat treatment resulted in protein unfolding, through denaturation, and release of protons and consequently, lower pH. Similarly, the pH of skim milk decreased immediately after UHT sterilization (Hansen and Melo, 1977; Venkatachalam et al., 1993).

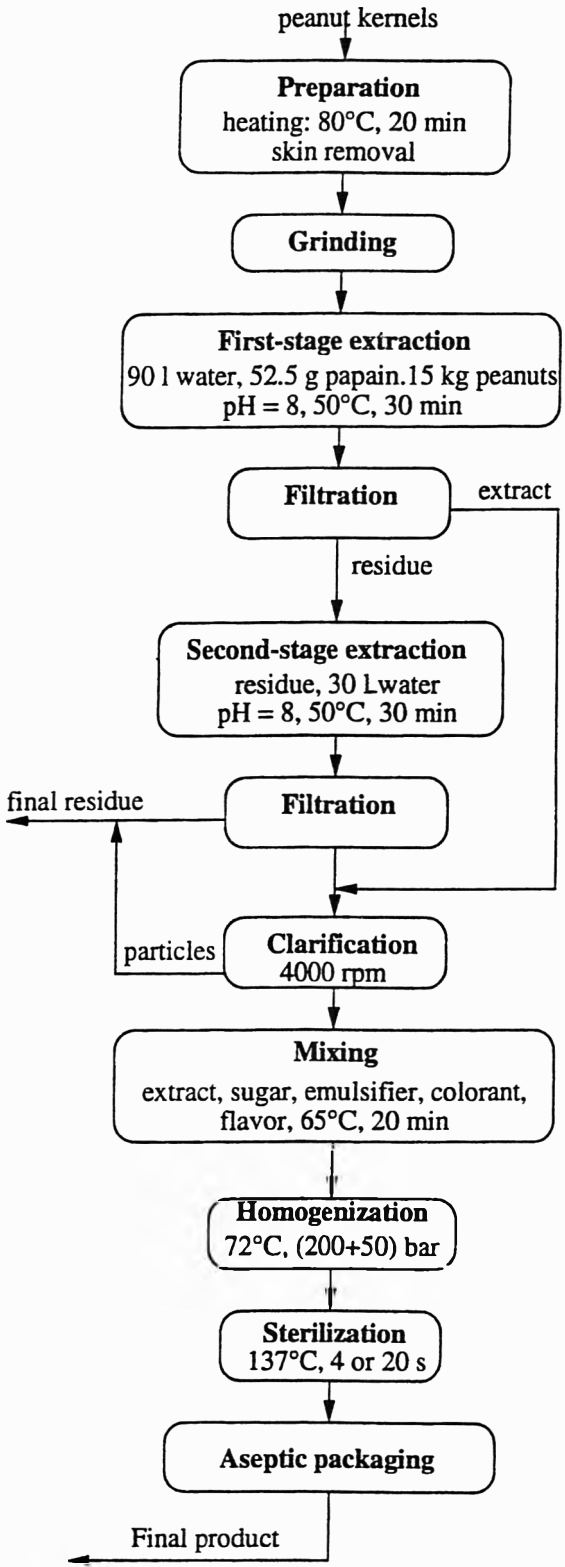

Fig. 1—Flow diagram for processing of 120 L peanut beverage.

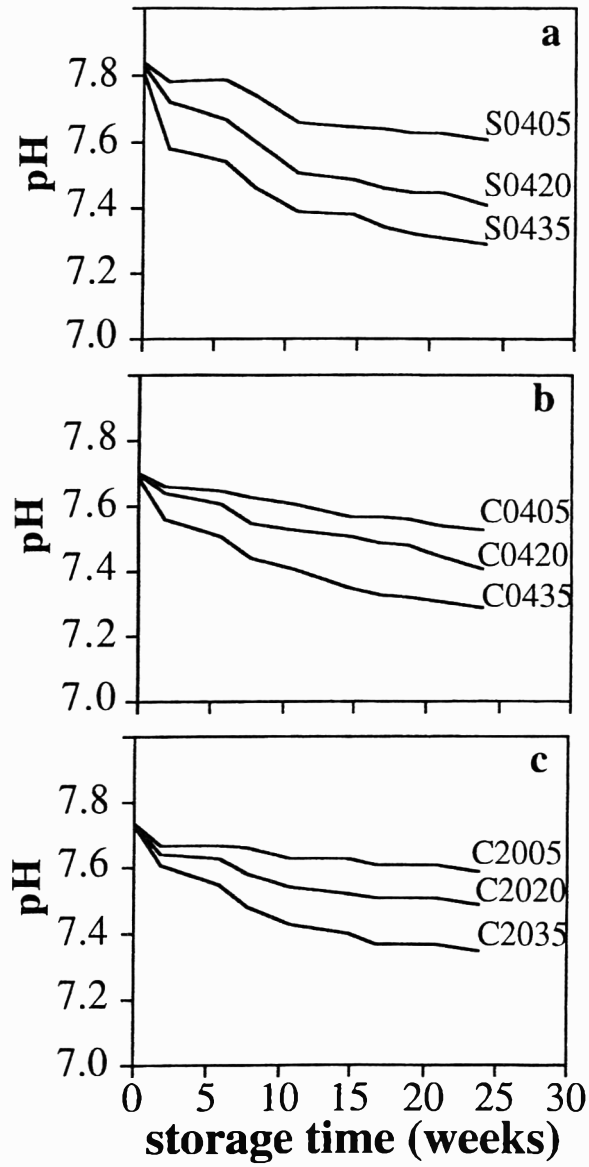

Fig. 2—Effect of storage temperature and time on pH of the beverages. See Table 1 for explanation of beverage codes.

Table 2—Homogenization index of beverages during storage

	Homogenization index (%)[b]						
	Storage time (wk)						
Beverage[a]	8	11	15	17	19	21	24
S0405	3.5	7.2	13.8	24.0	27.0	30.0	34.4
S0420	3.8	9.8	20.2	27.4	29.9	33.2	36.5
S0435	4.5	12.2	25.0	29.8	34.2	37.0	40.1
C0405	2.6	5.4	12.1	13.5	15.0	17.0	20.5
C0420	2.8	6.0	12.3	13.7	16.0	17.5	20.0
C0435	3.1	5.4	14.8	17.1	20.2	22.3	24.9
C2005	2.9	6.8	15.0	19.9	21.8	24.0	25.0
C2020	3.0	6.9	14.9	20.0	21.9	23.9	25.0
C2035	3.4	6.9	15.8	20.0	22.4	24.5	26.5

[a] See Table 1 for explanation of beverage codes.
[b] Means of two replicates ($0.0 \leq SD \leq 0.4$).

In general, there was a slight decrease in pH of strawberry-flavored (Fig. 2a) and chocolate-flavored beverages (Fig. 2b, c) during storage at 5, 20 and 35°C. The pH drop increased with increase in storage temperature. Also, statistical ANOVA (Table 4) showed that the storage temperature and time significantly influenced the pH drop. The observed drop in pH was not a consequence of microbial activity since no microbial growth was detected. It could be attributed to protein-protein reactions during storage, leading to release of free H^+.

Similar studies on UHT sterilized whole milk (Kocak and Zadow, 1985; Manji et al., 1986; Reddy et al., 1991) and on UHT soy beverage (Narayanan et al., 1993) revealed a continuous drop in pH during storage. Andrews et al. (1977) attributed a drop in pH of UHT milk stored at 30 and 37°C to loss of positive charges on the protein molecule. These were caused by involvement of free ε-NH_2 groups of amino acids and sugar in Maillard-type reactions. We were not sure if the same explanation for the pH drop (i.e., involvement in a Maillard-type

Table 3—Regression of sedimentation index (mL/12 mL) on storage time (wk)

Beverage[a]	Model constants (X 10 ^{3})[b] a (mL/12 mL)	b (mL/12 mL wk ^{1})	R^2
S040S	96	24	0.968
S042C	110	25	0.971
S043S	138	26	0.977
C040S	112	43	0.903
C042C	122	47	0.904
C0435	146	51	0.925
C2005	183	55	0.956
C2020	178	65	0.955
C2035	161	75	0.962

[a] See Table 1 for explanation of beverage codes.
[b] Model: sedimentation index = a + b (storage time).

reaction) was applicable. However, we found a significant ($P < 0.05$) correlation between decrease in beverage color lightness and pH drop during storage (Table 5).

Viscosity and extent of proteolysis. Changes in viscosity of beverages during storage were also compared (Fig. 3). The chocolate-flavored beverages were more viscous than the strawberry-flavored due to use of Recodan CM® that contained polysaccharide thickening agents (carrageenan and guar gum). Viscosity of the strawberry-flavored beverage was constant up to 6 mo storage at 5, 20 and 35°C. However, viscosity of the chocolate-flavored beverages changed during storage (Fig. 3a, b), and was affected by storage temperature and time (Table 4). Chocolate-flavored beverages stored at 35°C had a lower viscosity than those at 5°C and 20°C throughout storage. Thus, the higher storage temperature seemed to lower the rate of protein-solvent interactions leading to reduced swelling and, hence, lower viscosity.

Viscosity of the chocolate-flavored beverages stored at different temperatures considerably increased after 19 wk storage, indicating onset of age gelation. However, no formation of typical gel structure occurred, and the beverages had free-flowing characteristics. Age gelation has been described as coagulation, thixotropic gel formation, age thickening, partial gelation and lumpiness, characterised by a sudden and sharp increase in viscosity preceded by a period of constant viscosity (Venkatachalam et al., 1993). The gelation started at the same time in all beverages stored at 5, 20, and 35°C. This was in contrast to gelation in UHT milk where elevated storage temperatures accelerated the start of gelation (Kocak and Zadow, 1985). Age gelation of UHT stored milk has not been fully explained. However, two mechanisms have been suggested: It may be caused by proteolytic enzymes of indigenous or bacterial origin (Renner, 1988), or due to physicochemical processes such as protein polymerisation and conformational changes leading to aggregation of dissociated proteins (Venkatachalam et al., 1993).

Proteolysis was not significant (by paired t-test) in chocolate-flavored beverage C0435 ($P = 0.0003$) and beverage C2035 ($P = 0.0002$). Extent of proteolysis was 5.7% in beverage C0435 and 4.8% in beverage C2035. Heating them during extract preparation (50°C, 60 min), mixing (65°C, 20 min) and UHT sterilization seemed to inactivate proteolytic enzymes. Kroll and Klostermeyer (1984) found that preheating milk at 55 to 60°C for 20 min reduced the proteolytic activity by 45 to 50% in UHT milk. Therefore, we did not attribute gelation of the chocolate-flavored beverages to proteolysis. Also, Harwalkar et al. (1983) reported no appreciable change in extent of proteolysis in gelled sterilized canned evaporated milk stored at 28°C for 1 yr.

Microbial growth was not detected in beverages during storage. Also, decrease in pH during storage did not correlate with increase in viscosity of the chocolate-flavored beverages (Table 5). Therefore, we could not attribute gelation of the chocolate-flavored beverages to microbial activity. Similar observations were reported for UHT sterilized whole milk (Kocak and Zadow, 1985; Manji et al., 1986; Reddy et al., 1991) and evaporated milk (Harwalkar et al., 1983).

Protein polymerization through Maillard reaction was suggested as a mechanism leading to age gelation in UHT milk (Andrews, 1975). In our experiment, however, increase in viscosity did not significantly correlate with decrease in color lightness (Table 5). Similarly, De Koning and Kaper (1985) concluded that the Maillard reaction was not responsible for onset of gelation in UHT-treated concentrated casein micelle dispersions.

A possible cause of age gelation in chocolate-flavored beverages could be the interaction of peanut protein with polysaccharide thickening agents (carrageenan and guar gum) in Recodan CM® leading to aggregation of molecules. This explanation was justified by absence of gelation in the strawberry-flavored beverage where the emulsifier did not contain polysaccharide thickening agents.

Homogenization index. Emulsion stability of beverages during storage, expressed as homogenization index (HI) was compared (Table 2). HI values from 0 to 10% indicate excellent stability, between 11 to 20% good stability, and >20% poor stability (Anonymous, 1970). All beverages had excellent emulsion stability (HI = 0%) during the first 6 wk storage. However, stability was poor (HI > 20%) after 11 wk for the strawberry-flavored beverage S04, 19 wk for the chocolate-flavored beverage C04 and 17 wk in beverage C20. This was accompanied by visible fat separation. The emulsion stability decreased with increasing storage temperature and time (Table 2). The decrease in emulsion stability significantly correlated with pH drop (Table 5). This was attributable to partial loss of protein solubility which might result in higher aggregation rate and reduced emulsifying capacity of protein (Cheftel et al., 1985).

Sedimentation index. Stability of particles in the beverages during storage was assessed in terms of sedimentation index (accelerated sediment formation). At 0 wk, more sediment formed in the chocolate-flavored beverages (0.15–0.20 mL/12 mL) than in the strawberry-flavored one (0.10 mL/12 mL) after centrifugation. We attributed that to presence of insoluble chocolate particles. Also at 0 wk, more sediment formed in the chocolate-flavored beverage sterilized at 137°C for 20 sec (0.20 mL/12 mL) than in the one sterilized at 137°C for 4 sec (0.15 mL/12 ml). This could be caused by reduction in protein due to denaturation by heat. Similarly, Wilson et al. (1960) found that the sediment increased with severity of heat treatment of whole milk.

To follow the trend of sedimentation during storage, linear regression models:

$$S = a + bt$$

were fitted for the beverages, where S = sedimentation index (mL/12 mL), a = constant, b = sedimentation rate (mL/12 mL. wk^{-1}), t = storage time (wk). Table 3 lists values of a and b. All beverages had positive b values indicating that sediment formation increased with time. The dry weights of sediment from the chocolate-flavored and strawberry-flavored beverages were 92% and 96% protein, respectively. Therefore, increase in sedimentation index of the beverages during storage could be explained as follows: During UHT treatment, coagulae formed due to protein denaturation by heat, and upon storage the denatured insoluble proteins aggregated and formed sediment (after centrifugation).

Storage temperature and time had significant effects on sedimentation index (Table 4). Higher sedimentation rates occurred in beverages stored at 20°C and 35°C than in those stored at 5°C (Table 3). Also, sedimentation rates in stored UHT milk increased with increase in heat treatment and storage temperature (Ramsey and Swartzel, 1984). High correlation existed between pH drop and increase in sedimentation index in all beverages (Table 5). Peanut proteins were highly soluble in water at $2.0 > pH > 7.0$. However, solubility decreased after reducing pH from 7.0 to 6.0 (Rhee et al., 1973) or from pH 8.0 to pH 7.0 (Basha and Cherry, 1976). Therefore, the pH drop

Table 4—ANOVA for effect of storage temperature and time on physicochemical properties of beverages

		F - ratio[b]						
Parameter	Beverage[a]	pH	Viscosity	Homogenization index	Sedimentation index	Lightness	Hue	Chroma
Storage temperature	S04	699.5*	4.1	35.1*	55.7*	182.4*	0.7	1.9
	C04	318.4*	20.3*	24.4*	34.0*	144.3*	1.2	2.5
	C20	179.6*	26.7*	4.8	34.7*	53.7*	2.0	2.1
Storage time	S04	105.6*	1.6	415.8*	390.8*	2.6	9.7*	5.6*
	C04	44.9*	32.2*	306.2*	334.1*	1.3	37.7*	25.3*
	C20	18.3*	66.3*	1075.6*	192.6*	1.8	12.1*	7.9*

[a] See Table 1 for explanation of beverage codes.
[b] F - ratio has 2 and 44 degrees of freedom for storage temperature, and 9 and 44 degrees of freedom for storage time.
* Significant at 0.01 confidence level.

Table 5—Pearson product-moment correlation coefficients for physicochemical changes in beverages

		Parameter		
Parameter	Beverage[a]	pH	Viscosity	Homogenization index
Viscosity	S04	0.034		
	C04	−0.166		
	C20	−0.180		
Homog. index	S04	−0.721***	−0.087	
	C04	−0.699***	0.591***	
	C20	−0.592**	0.653***	
Sedim. index	S04	−0.769***	−0.089	0.978***
	C04	−0.700***	0.731***	0.950***
	C20	−0.725***	0.671***	0.948***
Lightness	S04	0.288*	−0.174	0.104
	C04	0.284*	−0.046	−0.190
	C20	0.353**	−0.008	−0.076

[a] See Table 1 for explanation of beverage codes.
* $p < 0.05$ ** $p < 0.01$ *** $p < 0.001$

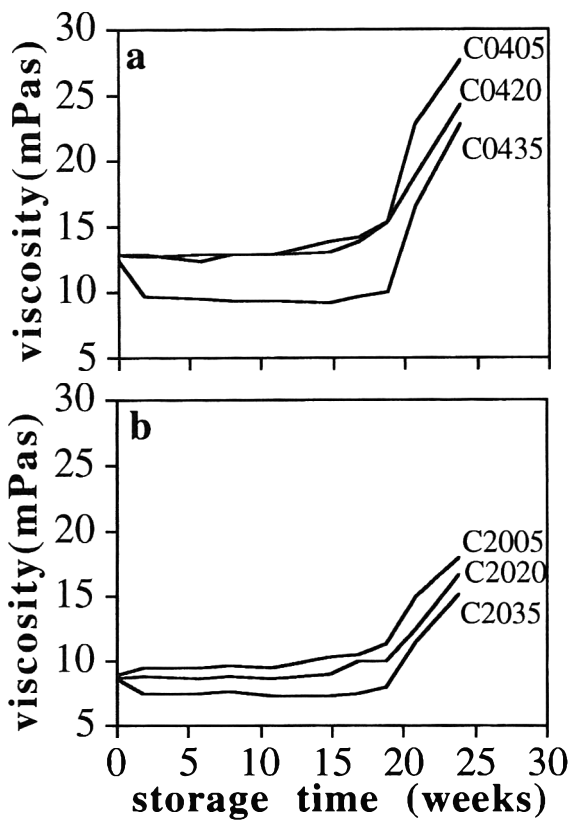

Fig. 3—Effect of storage temperature and time on viscosity of the beverages. See Table 1 for explanation of beverage codes.

during storage might have contributed to increase in sedimentation index. A high correlation existed (Table 5) between increase in viscosity and sedimentation index of gelled chocolate-flavored beverages. This could be explained by the fact that gelling of food proteins is preceded by protein unfolding, partial insolubilisation and aggregation (Cheftel et al., 1985).

Color. Color values L*, a* and b* for beverages during storage were also compared (Fig. 4). Lightness decreased after the first 6 wk at 5, 20 or 35°C by 30 to 37%, 39 to 53% and 43 to 60% respectively. However, between 6 and 24 wk, changes in lightness were negligible. Trend of change in lightness was an indication that Maillard reaction proceeded at relatively high rate during the first 6 wk. This was also reflected by a sharp increase in red component (+ a*). Change in lightness depended on storage temperature. The effect of storage time, however, was not significant (Table 4). Chocolate-flavored beverage C20 was darker than beverage C04. Also, all beverages became darker with increase in storage temperature, indicating increased rate of development of Maillard reaction browning products.

Age gelation reduced lightness of UHT whole milk due to increase in milk opacity (Reddy et al., 1991). However, in our experiment, gelation had no effect on lightness as demonstrated by nonsignificant correlation between changes in viscosity and in lightness (Table 5). No changes of any uniform patterns were observed in a* and b* for strawberry-flavored and chocolate-flavored beverages during storage (Fig. 4). Changes in hue and chroma were related to storage time only (Table 4).

CONCLUSIONS

CHANGES in the physicochemical properties of the beverages were minimal for storage up to 5 mos. In chocolate-flavored beverages, age gelation was the key factor that contributed to product quality deterioration. We attributed gelation to protein interaction with carrageenan and guar gum in Recodan CM®. Therefore, gelation onset could be delayed in chocolate-flavored beverages by replacing it with an agent which does not contain polysaccharide thickening agents. Break down of emulsion stability and sedimentation made negligible contributions to failure in product quality. Also, no visible sediment was observed in beverages (before centrifugation) at any storage temperatures for up to 6 mo. Microbiological quality remained high for >1 yr at all storage temperatures. Although the chocolate-flavored beverages stored at 37°C had lower viscosity, changes in the physicochemical properties proceeded at faster rates. Storage at refrigeration temperature (5°C) gave the best results. The UHT peanut beverage would be a suitable product in developing countries, since its quality remained good for up to 5 mos storage at 37°C.

REFERENCES

Andrews, A.T. 1975. Properties of aseptically packed ultra-high-temperature milk. III. formation of polymerised protein during storage at various temperatures. J. Dairy Res. 42: 89–99.
Andrews, A.T., Brooker, B.E., and Hobbs, D.G. 1977. Properties of aseptically packed ultra heat-treated milk. Electron microscopic examination of changes during storage. J. Dairy Res. 44: 283–292.
Andrews, G.R. 1986. Effect of storage, light and homogenization on the colour of ultra heat treated and sterilized milk. J. Dairy Res. 53: 615–624.
Anonymous. 1970. Svenska Mejeriernas Riksförening (SMR). *Analys Metoder for Mejerier*. AB Boktryck, Halsingborg, Sweden.

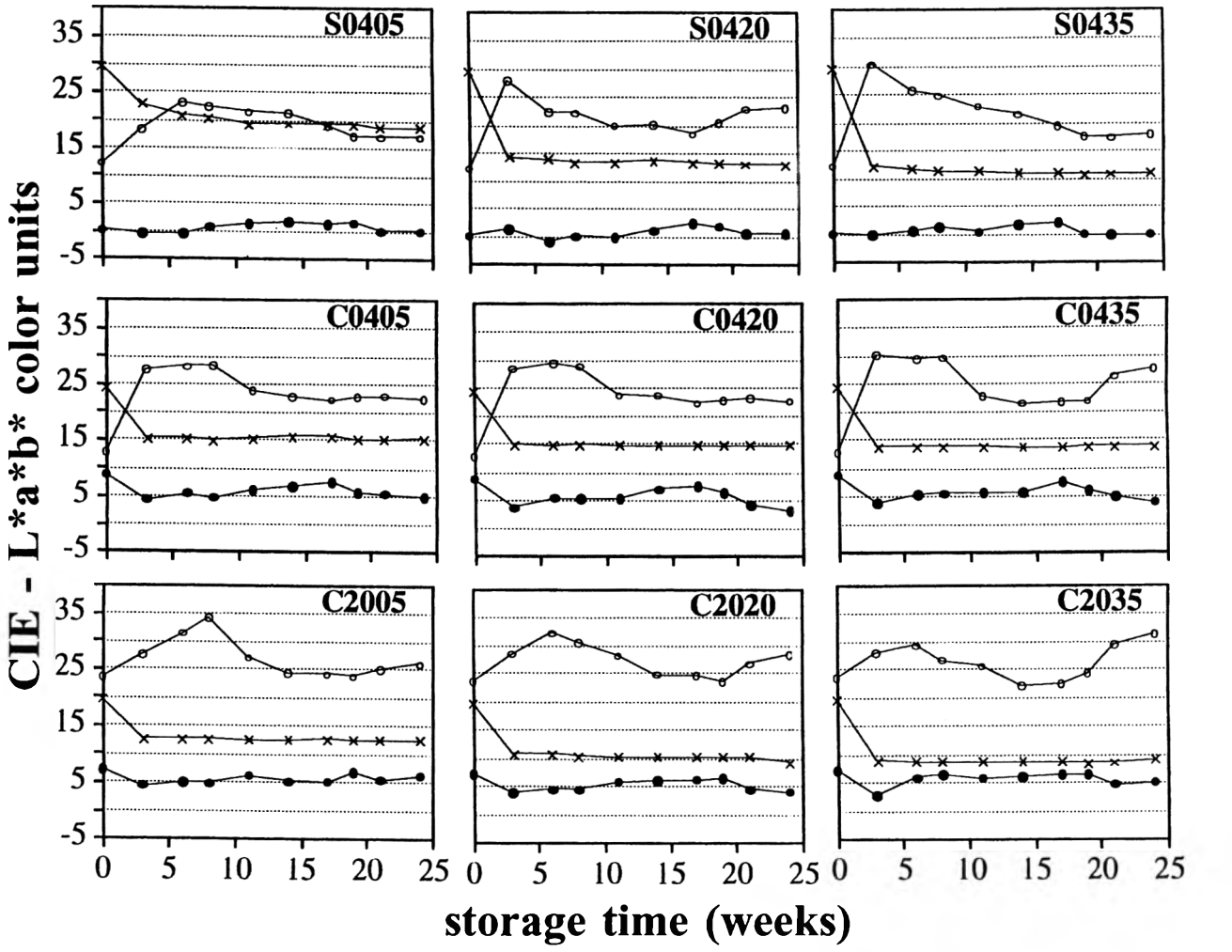

Fig. 4—Effect of storage temperature and time on color of the beverages. Color measured on CIE-L*a*b* coordinates: -X-L* (lightness), - ○ - a* (red - green), - ● - b* (yellow - blue). See Table 1 for explanation of beverage codes.

Anonymous. 1983. Laboratory tests. In: *Sterilab Instructions Book*. Reference no. N152545/0015A. Alfa-Laval Co., Lund, Sweden, section 5, pp. 5.

Basha, S.M.M. and Cherry, J.P. 1976. Composition, solubility, and gel electrophoretic properties of proteins isolated from florunner peanut seeds. J. Agric. Food Chem. 24: 359–365.

Burton, H. 1988. *Ultra-High-Temperature Processing of Milk and Milk Products*. (Ed) H. Burton. Elsevier Applied Science Publishers Ltd, England.

Cheftel, J.C., Cuq, J.-L. and Lorient, D. 1985. Amino acids, peptides, and proteins. Ch. 5, In *Food Chemistry*, 2nd ed., Owen R. Fennema (Ed.), p. 245–370. Marcel Dekker, Inc, New York.

De Koning, P.J. and Kaper, J. 1985. Effects of some proteinase inhibitors and of the Maillard reaction on the processes of age-thinning and gelation of UHTST-sterilized concentrated casein micelle dispersions. Neth. Milk Dairy J. 39: 37–47.

Hansen, A.P. and Melo, T.S. 1977. Effect of ultra-high-temperature steam injection upon constituents of skim milk. J. Dairy Sci. 60: 1368–1373.

Harwalkar, V.R. and Vreeman, H.J. 1978. Effect of added phosphates and storage on changes in ultra-high temperature short-time sterilized concentrated skim-milk. 1. viscosity, gelation, alcohol stability, chemical and electrophoretic analysis of proteins. Neth. Milk Dairy J. 32: 94–111.

Harwalkar, V.R., Beckett, D.C., McKellar, R.C., Emmons, D.B., and Doyle, G.E. 1983. Age-thickening and gelation of sterilized evaporated milk. J. Dairy Sci. 66: 735–742.

IDF. 1987. Enumeration of microorganisms—Colony count at 30°C. International Dairy Federation. IDF Standards 100A: 1987. Brussels, Belgium.

IDF. 1990. Enumeration of yeasts and moulds—Colony count technique at 25°C. International Dairy Federation. IDF Standards 94B: 1990. Brussels, Belgium.

Kocak, H.R. and Zadow, J.G. 1985. Age gelation of UHT whole milk as influenced by storage temperature. Aust. J. Dairy Technol. 40(1): 14–21.

Kroll, S. and Klostermeyer, H. 1984. Heat inactivation of exogenous proteinases from Pseudomonas fluorescens. Z. Lebensm. Unters. Forsch. 179: 288–295.

Manji, B., Kakuda, Y., and Arnott, D.R. 1986. Effect of storage temperature on age gelation of ultra-high temperature milk processed by direct and indirect heating systems. J. Dairy Sci. 69: 2994–3001.

Mogensen, G. and Poulsen, P.R. 1980. Quality of UHT milk stored at refrigerated and ambient temperatures as compared to HTST pasteurized milk. Milchwissenschaft 35: 552–556.

Narayanan, K.R.A., Kumar, A., and Patil, G.R. 1993. Kinetics of various deteriorative changes during storage of UHT soy beverage and development of a shelf-life prediction model. Lebensm. Wiss. Technol. 26: 191–197.

Ramsey, J.A. and Swartzel, K.R. 1984. Effect of ultra high temperature processing and storage conditions on rates of sedimentation and fat separation of aseptically packaged milk. J. Food Sci. 49: 257–262.

Reddy, K.K., Nguyen, M.H., Kailasapathy, K., and Zadow, J.G. 1991. The effect of some treatments and storage temperatures on UHT whole milk. Aust. J. Dairy Technol. 46(2): 57–63.

Renner, E. 1988. Storage stability and some nutritional aspects of milk powders and ultra high temperature products at high ambient temperatures. J. Dairy Res. 55: 125–142.

Rhee, K.C., Cater, C.M., and Mattil, K.F. 1973. Effect of processing pH on the properties of peanut protein isolates and oil. Cereal Chem. 50: 395–404.

Rustom, I.Y.S., López-Leiva, M.H., and Nair, B.M. 1991a. A study of factors affecting extraction of peanut solids with water. Food Chem. 42: 153–165.

Rustom, I.Y.S., López-Leiva, M.H., and Nair, B.M. 1991b. Optimization of extraction of peanut proteins with water by response surface methodology. J. Food Sci. 56: 1660–1663.

Rustom, I.Y.S., López-Leiva, M.H., and Nair, B.M. 1993. Extraction of peanut solids with water—effect of the process and enzymatic hydrolysis. Food Sci. Techno.. (LWT) 26: 72–75.

STATGRAPHICS. 1991. *Statistical Graphics System. Version 5.0. Reference Manual.* Statistical Graphics Corp., Princeton, NJ, USA.

Venkatachalam, N., McMahon, D.J., and Savello, P.A. 1993. Role of protein and lactose interactions in the age gelation of ultra-high temperature processed concentrated skim milk. J. Dairy Sci. 76: 1882–1894.

Wilson, H.K., Herreid, E.O., and Whitney, R.M. 1960. Ultracentrifugation studies of milk heated to sterilization temperatures. J. Dairy Sci. 43: 165–174.

Zadow, J.G. 1993. Development of UHT in Australia. Food Australia 45: 274–277.

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Flavor Characteristics of Lactic, Malic, Citric, and Acetic Acids at Various pH Levels

PAM HARTWIG and MINA R. McDANIEL

ABSTRACT

The objective of this study was, through the use of free-choice profiling, to determine flavor characteristics of four acids (citric, malic, lactic, acetic) and two acid blends (lactic/acetic 1:1 and 2:1) at three different pH levels (3.5, 4.5, 6.5) and at 0.2% (w/v). Research was conducted to explore flavor differences and similarities among common food acidulants. Generalized Procrustes Analysis was performed on free-choice profiling data, which resulted in three significant principal axes. The first principal axis was characterized by overall intensity and sourness, the second by vinegar and saltiness, and the third by astringency.
Key Words: sensory, free-choice-profiling, acidity, flavor characteristics

INTRODUCTION

ONE OF THE MOST functional ingredients in the food industry is acids (Andres, 1985), contributing to a wide variety of properties in foods. Acids can control pH, preserve food, provide leavening, aid in gel formation, prevent nonenzymatic browning, act as a synergist for antioxidants, chelate metal ions, and most importantly, add and enhance flavor. Acids are found in most types of food products, such as jams, jellies, preserves, bakery, dairy, and meat products, beverages, and confectionery products. Many acidulants occur naturally in foods, in cells of plants and animals and thus most are generally recognized as safe (GRAS listed).

A trend for use of acidulants is the combination of two or more acids in formulated food products. In nature one acid is rarely found alone; hence, combining acids enables an acidified food to more readily simulate natural flavor. Two acids frequently found in combination are lactic and acetic. In preparing pickles and relishes, lactic acid can be added to vinegar to provide milder, more subtle taste sensations (Gardner, 1972; Fabian and Wadsworth, 1939). In salad dressings and marinades, lactic/acetic blends have been found microbiologically to act synergistically; they are especially inhibitory to outgrowth of heterofermentative lactobacilli (Doores, 1990).

Acidulants have one shared sensory characteristic: sourness. However, acids are different in degree of sourness and in non-sour aroma and flavor characteristics. Few studies have profiled acid flavor. For example, Arnold (1975) and Pszczola (1988) proposed that intensity and duration of acidic taste differed among acids. Straub (1992) found differences in power functions and time-intensity curves for sourness and astringency of 7 organic acids and one inorganic acid. Rubico (1993) and Rubico and McDaniel (1992) evaluated flavor profiles of several organic acids and their blends, and inorganic acids, and found that acids could greatly differ in sensory character ie. sourness, bitterness, and astringency. Noble et al. (1986) studied organic acids in binary acid solutions and reported that acids at equal pH or equal titratable acidity varied in sourness intensity. While most studies have focused on sourness, published research has shown that acids have non-sour characteristics as well, i.e. bitterness and astringency (Straub, 1992; Rubico, 1993; Rubico and McDaniel, 1992).

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Depending upon pH of the final product, the flavor profile of acids can vary significantly This is a result of the acid changing chemical form according to its pKa. We sought to evaluate acids on an equal weight and equal pH basis in order to compare/contrast resulting flavors. In addition, the flavor of a given acid can change depending upon pH level; hence, three different pH levels were studied. By knowing differences in acid flavor at a given pH, acidified food products could be formulated to attain specified characteristics. Our primary objective was to determine flavor similarities and differences of common organic food acidulants and acid blends at several equivalent pH levels and on an equivalent concentration basis.

MATERIALS & METHODS

Samples

Eighteen acid solutions (four acids and two blends at three pH levels) were evaluated. Anhydrous citric and DL-malic acids were obtained from Haarmann and Reimer (H&R) Company (Elkhart, IN), glacial acetic acid (U.S.P.-F.C.C.) from J.T. Baker Inc. (Phillipsburg, NJ), and L(+)Lactic acid (88%) from PURAC America, Inc. (Lincolnshire, IL). In addition to the four acids, two acid blends were prepared: lactic/acetic (1:1 and 2:1). Acid solutions were evaluated at 0.2% (w/v); this concentration was chosen based on preliminary sensory evaluations to attain a reasonable intensity at all pH levels tested. The acid solutions were adjusted to pH 3.5, 4.5, and 6.5. These levels were selected to investigate acid characteristics at specific pH levels often used for formulated food systems. Salad dressings, canned vegetables, and seafood salads are examples at pH 3.5, 4.5 and 6.5, respectively.

In preparation of acid solutions for each panel session, 0.2% (w/v) of acid was added to most of the water and then adjusted with sodium hydroxide (NaOH) to the appropriate pH level. After titration, acid solutions were filled to volume with amounts of water needed to ensure equi-concentration of acid in all solutions. Acids were diluted with fresh deionized, distilled water (Milli-Q Reagent Water System, Millipore Corporation, Bedford, MA).

pH and titratable acidity

NaOH pellets (U.S.P.-F.C.C.) purchased from J.T. Baker Inc. (Phillipsburg, NJ) were used to prepare titrating solutions. A stock solution was made by stirring the pellets and deionized, distilled water [1:1 (w/w)]. After making the stock solution, one dilution step was performed to prepare a ~0.35N NaOH solution. This solution was used to adjust pH of the acid solutions to 3.5, 4.5, and 6.5. The pH levels were measured by a pH electrode with a microprocessor pH/mV meter (Corning Model 125, Medfield, MA) equipped with a combination pH electrode (Sensorex Model S200C, Stanton, CA). Using the same pH meter titratable acidity was measured to end-point pH 8.2 with ~0.085 N NaOH.

Panelists

Seven male and five female students and staff from the Dept. of Food Science & Technology at Oregon State Univ. served as panel members. Eight members had previous panel experience.

Presentation of samples

Samples were presented at room temperature (~23°C) in coded 57 mL plastic cups. Each cup contained ~30 mL of sample. Panelists were instructed to take a comfortable and consistent amount when evaluating each sample. All training and testing sessions were limited to a single pH level. This was done in order to maximize acid comparisons within

Table 1—Terms generated by each panelist in the free-choice profiling of acids

	Pan 1	Pan 2	Pan 3	Pan 4	Pan 5	Pan 6	Pan 7	Pan 8	Pan 9	Pan 10	Pan 11	Pan 12
1.	OI	OI	OI	OI	OI	OI	OI	OI	OI	OI	OI	OI
2.	Sour	Sour	Sour	Sour	Sour	Sour	Sour	Sour	Sour	Sour	Sour	Sour
3.	Astrin.	Astrin.	Astrin.	Astrin.	Astrin.	Astrin.	Astrin.	Astrin.	Astrin.	Astrin.	Astrin.	Astrin.
4.	Salty	Salty	Salty	Salty	Salty	Salty	Salty	Salty	Salty	Salty	Salty	Salty
5.	Bitter	Bitter	Bitter	Bitter	Bitter	Bitter	Bitter	Bitter	Bitter	Bitter	Bitter	Bitter
6.	Sweet	Sweet	Sweet	Sweet	Sweet	Sweet	Sweet	Sweet	Sweet	Sweet	Sweet	Sweet
7.	OI*	Metallic	Vinegar	Citrus	Metallic	Vinegar	Soapy	Vinegar	Citrus	OI*	Tart	Vinegar
8.	Sour*	Soapy	OI*	Vinegar	Fruity	OI*	OI*	OI*	Soapy	Sour*	Lime	Lemon
9.	Astrin.*	Citrus	Sour*	OI*	Vinegar	Sour*	Sour*	Sour*	Vinegar	Astrin.*	Soapy	Dirty
10.	Bitter*	Vinegar	Astrin.*	Sour*	OI*	Astrin.*	Astrin.*	Astrin.*	OI*	Bitter*	OI*	Tart
11.		OI*	Bitter*	Astrin.	Sour*	Bitter*	Bitter*	Bitter*	Sour*	Soapy*	Sour*	OI*
12.		Sour*		Bitter*	Astrin.*			Vinegar*	Astrin.*		Astrin.*	Sour*
13.		Astrin.*		Salty*	Bitter*			Salty*	Bitter*		Bitter*	Astrin.*
14.		Bitter*			Salty*			Salty*			Tart*	Bitter*
15.		Salty*			Soapy*			Soapy*			Vinegar*	
16.		Soapy*			Vinegar*			Vinegar*			Salty*	
17.		Vinegar*										

* Denotes after expectoration.

Table 2—Principal axis 1: mean scores for different acids

Acid/Acid mixture[k]	pH level	Mean score (Principal Axis 1)
Lactic	6.5	0.377[a]
Malic	6.5	0.367[a]
Lactic/Acetic (1:1)	6.5	0.350[a]
Lactic/Acetic (2:1)	6.5	0.347[a]
Citric	6.5	0.340[a]
Acetic	6.5	0.310[ab]
Lactic	4.5	0.260[b]
Citric	4.5	0.110[c]
Malic	4.5	0.013[d]
Lactic/Acetic (2:1)	4.5	−0.047[d]
Citric	3.5	−0.127[e]
Lactic/Acetic (1:1)	4.5	−0.167[ef]
Lactic	3.5	−0.267[fg]
Malic	3.5	−0.260[g]
Acetic	4.5	−0.273[g]
Lactic/Acetic (2:1)	3.5	−0.370[h]
Lactic/Acetic (1:1)	3.5	−0.447[i]
Acetic	3.5	−0.563[j]

[a-j] Different letter superscripts indicate significant differences at $p<0.05$ for each column separated by Least Significant Difference (LSD).
[k] All acids at 0.2% (w/v).

each pH level, rather than measuring differences due to pH changes. Due to similar intensities of acid solutions within each pH level, character differences were more discernible by this presentation. At each session four acids and two blends at a given pH level were randomly presented and evaluated. One replication (18 samples) was completed within 1 week (three sessions/wk). Three replications were completed.

Training

The concept of free-choice profiling (FCP) was introduced to panelists on the second of eight practice sessions. During the first two sessions, different concentrations of citric acid, sodium chloride, sucrose, caffeine, and alum were used for identification of, or introduction to, the four basic tastes (sour, salty, sweet, bitter) and astringency, respectively. They were also presented to practice rating the magnitude of different characteristics on a 16-point intensity scale (0 = none, 7 = moderate, 15 = extreme), which was used throughout the study. For the first few practice sessions, panelists were asked to list all sensory characteristics which described the perceived attributes of the samples. Later, these terms were used to develop a ballot for subsequent testing. To enhance interpretation, panelists were asked to define their own terms. For testing sessions, panelists were seated in separate, well ventilated booths with incandescent lighting.

When evaluating acid samples, the 'sip-and-spit' method was applied. Panelists were instructed to sip the sample and manipulate in the mouth for 5 sec, then expectorate. They were asked to taste samples in a natural fashion. While not instructed to smell samples, acetic acid was perceived, in part, retronasally. Panelists rated individual descriptors on intensity perceived while in the mouth, and then rated descriptors again after expectoration. Panelists were instructed to rinse with water between samples.

Statistical analysis

Generated data were analyzed by generalized procrustes analysis (GPA) using Procrustes-PC Version 2.0 (Dijksterhuis and van Buuren, 1989) and by Statistical Analysis System for Personal Computer (SAS Institute, Inc., 1987, Cary, NC). For the FCP analysis, data from each of the 12 panelists were assembled into matrices of 54 rows (six solutions at three pH levels assessed over three replications) by n columns where n represented the number of attributes for each panelist. Using these matrices GPA was performed. GPA produces a consensus sample configuration, showing interrelationships between samples and descriptors. Analysis of variance (ANOVA) on the principal axis scores was used to determine significant differences among samples and, where appropriate, least significant differences ($p \leq 0.05$). For GPA acids were analyzed as 18 unstructured solutions to determine whether clusters in the axis space would reflect the factorial structure (six acids at three pH levels).

RESULTS & DISCUSSION

Sensory results

FCP of the samples generated between 10 (Panelist 1) and 17 (Panelist 2) descriptors with an average of 13 descriptors/panelist (Table 1). Panelists used 10 descriptors in common: Overall Intensity (OI), Sour, Astringency (Astr), Salty, Bitter, Sweet, OI*, Sour*, Astr*, Salty* [* denotes after expectoration]. Vinegar (7), Vinegar* (5), Citrus (5), and Salty*(6) were common additional terms used by several panelists (number of panelists noted in parentheses).

ANOVA on consensus scores from GPA determined that the first three Principal Axes (PA) were significant. Tables 2, 3, and 4 show first, second, and third PAs, respectively). For GPA results Fig. 1 for first PA vs second PA and Fig. 2 for the first PA vs third PA. Triangles represent the three replications, where size of triangles illustrates replication variability with each sample. Thus, the smaller the area of the triangle, the better the panelists replicated and discriminated the sample. Combinations of descriptors for each panelist characterized the axes, where the most important descriptors had the highest loadings (Table 5).

The first three PAs explained nearly 86% of the variation as 72%, 8%, and 6% were explained by the 1st, 2nd and 3rd PAs, respectively. The high percentage explained was due to large and obvious differences resulting when evaluating acids at different pH levels. The first PA was characterized by overall intensity and sourness as these descriptors created high loadings for every panelist (Table 5). Astringency was also important on this PA for half the panel. At all pH levels, acetic acid was the most sour and intense; lactic and citric acids were the least (Fig. 1). These findings confirm research by Pangborn (1963) and CoSeteng et al. (1989). They found sourness intensity was a function of chemical structure. CoSeteng et al. (1989) found the number of carboxylic groups was related to sourness intensity

Table 3—Principal axis 2: mean scores for different acids

Acid/Acid Mixture[j]	pH level	Mean score (Principal Axis 2)
Acetic	4.5	0.127[a]
Acetic	3.5	0.090[ab]
Lactic/Acetic (1:1)	4.5	0.080[ab]
Acetic	6.5	0.053[bcd]
Lactic/Acetic (1:1)	3.5	0.040[cd]
Lactic/Acetic (1:1)	6.5	0.033[cde]
Lactic/Acetic (2:1)	6.5	0.033[cde]
Malic	6.5	0.021[def]
Citric	6.5	0.010[defg]
Malic	4.5	−0.013[efg]
Lactic/Acetic (2:1)	4.5	−0.014[efg]
Lactic	6.5	−0.019[fg]
Lactic	4.5	−0.027[fg]
Lactic/Acetic (2:1)	3.5	−0.030[g]
Citric	4.5	−0.033[g]
Malic	3.5	−0.096[h]
Citric	3.5	−0.103[h]
Lactic	3.5	−0.153[i]

[a-i] Different letter superscripts indicate significant differences at $p<0.05$ for each column separated by Least Significant Difference (LSD).
[j] All acids at 0.2% (w/v).

Table 4—Principal axis 3: mean scores for different acids

Acid/Acid Mixture[i]	pH level	Mean score (Principal Axis 3)
Acetic	4.5	0.063[a]
Lactic/Acetic (1:1)	4.5	0.057[a]
Malic	4.5	0.053[ab]
Malic	3.5	0.050[abc]
Citric	4.5	0.047[abcd]
Citric	3.5	0.040[abcde]
Lactic/Acetic (2:1)	4.5	0.022[abcdef]
Lactic	4.5	−0.005[bcdefg]
Lactic	6.5	−0.014[bcdefg]
Acetic	6.5	−0.014[bcdefgh]
Lactic/Acetic (1:1)	6.5	−0.016[cdefgh]
Acetic	3.5	−0.019[cdefgh]
Malic	6.5	−0.020[defgh]
Lactic/Acetic (2:1)	6.5	−0.020[defgh]
Citric	6.5	−0.027[efgh]
Lactic/Acetic (2:1)	3.5	−0.046[fgh]
Lactic	3.5	−0.060[gh]
Lactic/Acetic (1:1)	3.5	−0.083[h]

[a-h] Different letter superscripts indicate significant differences at $p<0.05$ for each column separated by Least Significant Difference (LSD).
[i] All acids at 0.2% (w/v).

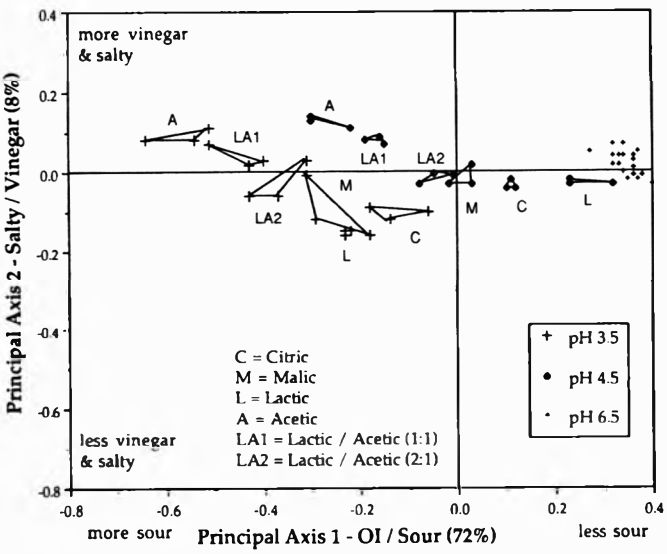

Fig. 1—Sample consensus plot for free-choice profiling of different acids/acid blends (0.2% (w/v)) following generalized procrustes analysis: principal axes 1 vs 2. Three points represent three replications across 12 panelists.

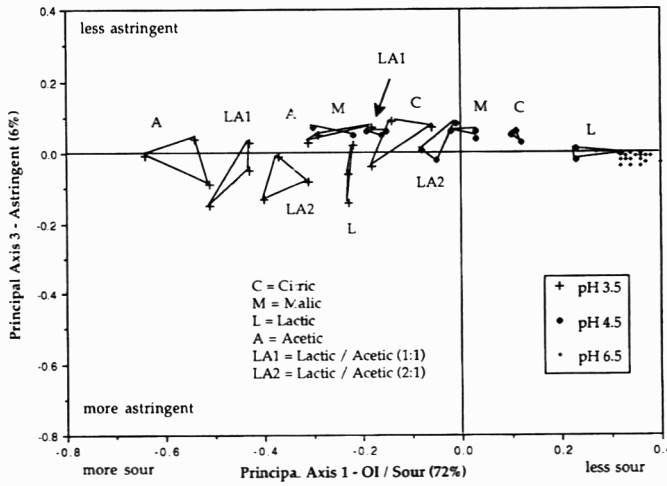

Fig. 2—Sample consensus plot for free-choice profiling of different acids/acid blends (0.2% (w/v)) following generalized procrustes analysis: principal axes 1 vs 3. Three points represent three replications across 12 panelists.

(ie. in decreasing order of sourness: mono-carboxylic acids (CA)>>di-CAs>>tri-CAs). In addition, they reported molecular weight and polarity were important factors in sourness perception, demonstrating that increasing molecular weight and hydrophobicity of an acid molecule increased sourness intensity.

With few exceptions, the relative order of the acids remained constant at each pH level. Acetic acid and then the lactic/acetic blends had the lowest PA scores for every pH level as PA 1 descriptors with the highest loadings were on the negative side of the axis. Overlap occurred between pH levels as acetic acid at pH 4.5 was significantly more sour than citric acid at pH 3.5.

The size of triangles (showing the three replications) provides useful information. For example, at pH 3.5 the relatively large area of malic acid illustrated the difficulty that panelists had in replicating perceived differences. Though the area of malic acid was larger than others, no overlap occurred. In contrast, the close proximity of lactic acid at pH 3.5 exemplified good consensus among panelists. This contradicted previous research reports that found lactic acid relatively difficult to discriminate as shown by large replication variation (CoSeteng et al., 1989; Straub, 1992). Differences could be due to training regimen and/or source of lactic acid.

Flavor intensity was important in sample discrimination, regardless of the fact that different pH levels were evaluated at separate sessions. Acids at pH 3.5 were all very intense; thus, these samples were more readily separated due to greater differences in intensity. However, as pH increased, the intensity decreased as did intersample distances. As a result, at pH 6.5 intensities of all samples were very weak and some were barely perceivable. Thus, all were contained in one general area (Fig. 1).

The second PA was characterized by vinegar and saltiness. For the seven panelists who used the term vinegar, this descriptor received high loadings for their second PA. Saltiness also received high loadings from over half the panelists (1, 2, 4, 6, 8, 10, and 11) [Table 5]. At pH 6.5, sourness was diminished, leaving only minor qualities such as saltiness. This was expected since the NaOH for titration was at the highest concentration in the acid solutions at the highest pH levels (Table 6).

Most acetic acid and lactic/acetic blends were on the positive side of the axis, as were acetic, malic and citric acids at pH 6.5 (Table 3). Lactic acid was the least salty/vinegar at all pH levels, and the least amount of NaOH was added to achieve the 3 levels relative to other acids (Table 6). The good replications of acetic and lactic/acetic blends were due to the distinct vinegar character from acetic acid, which characterized the 2nd axis.

The third PA was characterized by astringency (Table 5) with high loadings received from all but one panelist. Separation on

Table 5—Loadings[a] of the descriptors for the first three principal axes following free-choice profiling for acids at three pH levels

Panelist No.	Principal Axis 1	Principal Axis 2	Principal Axis 3
1	-Ol(0.59) -Sour(0.56) -Ol*(0.38) -Sour*(0.30)	Ol(0.36) +Salty(0.63) +Sweet(0.43) -Astrin*(0.35)	-Astrin(0.74) -Astrin*(0.55)
2	-Ol(0.55) -Sour(0.59)	Ol(0.34) -Astrin(0.33) +Salty(0.55) +Vinegar(0.34)	-Astrin(0.65) -Salty(0.35) -Astrin*(0.32)
3	-Ol(0.61) -Sour(0.63)	-Astrin(0.48) -Sweet(0.37) +Vinegar(0.67) -Astrin*(0.33)	-Astrin(0.49) -Sweet(0.65) -Astrin*(0.41)
4	-Ol(0.54) -Sour(0.55) -Astrin(0.33) -Ol*(0.32)	Salty(0.36) +Vinegar(0.73) -Citrus(0.46)	Sour(0.41) -Astrin(0.43) -Bitter(0.30) +Vinegar(0.40) +Citrus(0.40) -Bitter*(0.32)
5	-Ol(0.56) -Sour(0.66)	-Astrin(0.33) +Vinegar(0.63) -Fruity(0 54)	-Astrin(0.66) -Bitter(0.34) +Sweet(0.39)
6	-Ol(0.43) -Sour(0.47) -Astrin(0.43) -Ol*(0.30) -Sour*(0.32) -Astrin*(0.30)	Salty(0.67) -Sweet(0.36) +Vinegar(0.56)	Sour(0.38) -Astrin(0.50) +Salty(0.45) -Vinegar(0.39) -Bitter*(0.33)
7	-Ol(0.51) -Sour(0.72) -Astrin(0.31)	-Astrin(0.53) -Bitter(0.33) +Sour*(0.49) -Astrin*(0.36)	-Bitter(0.32) -Ol*(0.45) -Sour*(0.49) -Astrin*(0.46) -Bitter*(0.31)
8	-Ol(0.49) -Sour(0.42) -Astrin(0.40) Vinegar(0.49)	Salty(0.79)	Sour(0.37) -Astrin(0.53) +Sour*(0.67)
9	-Ol(0.43) -Sour(0.55) -Astrin(0.30) -Sour*(0.34) -Vinegar*(0.35)	Vinegar(0.55) -Sour*(0.39) +Vinegar*(0.58)	Sour(0.48) -Astrin(0.77) -Vinegar(0.31)
10	-Ol(0.66) -Sour(0.70)	Salty(0.35) +Bitter(0.57) -Sour*(0.33) -Astrin*(0.58)	-Astrin(0.86)
11	-Ol(0.55) -Sour(0.56)	Salty(0.90)	-Astrin(0.52) +Sour*(0.42) -Astrin*(0.64)
12	-Ol(0.54) -Sour(0.52) -Astrin(0.43) -Sour*(0.32)	-Astrin(0.52) +Vinegar(-0.58) -Astrin*(0.33) +Vinegar*(0.49)	-Ol(0.35) -Vinegar(0.31) -Lemon(0.38) +Ol*(0.49) +Sour*(0.49)

[a] Descriptors with loadings <0.30 were not included in the table.
* Denotes after expectoration.

Table 6—Means and standard deviations of NaOH added in % (w/v) to acids to achieve three pH levels

	pH levels		
Acid	3.5	4.5	6.5
Citric	0.0385[a] (0.0077)[b]	0.0682 (0.0029)	0.1250 (0.0040)
Malic	0.0300 (0.0060)	0.0738 (0.0022)	0.1218 (0.0078)
Lactic	0.0211 (0.0042)	0.0619 (0.0052)	0.0727 (0.0012)
Acetic	0.0030 (0.0006)	0.0486 (0.0068)	0.1368 (0.0061)
L/A (1:1)	0.0102 (0.0020)	0.0510 (0.0014)	0.1017 (0.0033)
L/A (2:1)	0.0137 (0.0027)	0.0510 (0.0034)	0.0883 (0.0030)

[a] Mean.
[b] Standard deviation.

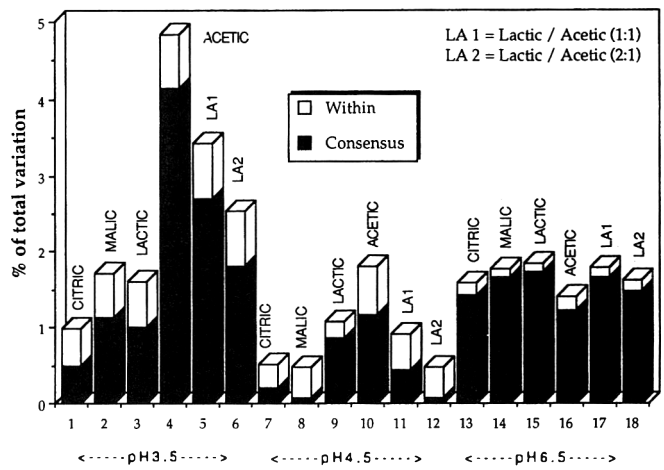

Fig. 3—Percentages consensus and within (residual) variation distributed over 18 samples.

this axis was by pH, as acids at pH 4.5 were apparently less astringent than those at pH 3.5 and 6.5. At pH 3.5, lactic acid was the most astringent, significantly more so than citric or malic. At pH 4.5, lactic was also the most astringent, significantly more than all others. At pH 6.5, none of the acids elicited significantly different astringent sensations. Marked astringency differences in acids were cited by Rubico and McDaniel (1992) and Straub (1992).

The ASTM Committee on Sensory Evaluation of Materials and Products has defined astringency as "the complex of sensations due to shrinking, drawing or puckering of the epithelium as a result of exposure to substances such as alums or tannins" (ASTM 1993). While historically it has been considered of equal importance to the four basic tastes (Bartoshuk, 1978), the chemical reactions promoting astringency in acids are still not well understood. One hypothesis by Lawless et al. (1994) is that astringency could originate from one of two sources: from the direct contribution of H^+ ions or from the hydrogen bonding capabilities of the hydroxyl groups on the anion or the dissociated acid. They proposed that the greater the number of hydroxyl groups, the more astringent the solution should be. Another more common hypothesis is that acids precipitate proteins in saliva or cause sufficient conformational changes so that lubrication is lost when acids form complexes with salivary proteins or mucopolysaccharides (Bate-Smith, 1954, 1973). Neither of these would explain the apparent high astringency of lactic acid.

Panelist and sample variation

Panelists' individual performances were examined by the percentage residual variation data from GPA. None of the panelists had residuals that were significantly greater than the others; therefore, residual data are not shown. The percentage consensus and percentage within (residual) (Fig. 3) were related to acids as well as pH level. The lower, darker portion of the histograms represents the percentage consensus variation, and the upper, lighter portion is the within (residual) variation for various acids. The extent of discrimination among samples was related to intensity. Panelists had the greatest consensus for acetic and lactic/acetic blends at pH 3.5. This confirmed CoSeteng et al. (1989) findings that acetic acid produced the most consistent results with varying pH levels. Acids at pH 3.5 were the most intense, making differences between samples more apparent. At pH 4.5, the lowest consensus scores for all acids were found. Generally, these acids were slight to moderate intensity. The low consensus scores at that level indicated that those samples were most dif-

Table 7—Means and standard deviations on titratable acidity measurements of acids

Acid	pH Level 3.5	4.5	6.5	
Citric	0.1454[a] (0.0005)[b]	0.0995 (0.0003)	0.0130 (0.0000)	
Malic	0.1403 (0.0006)	0.0716 (0.0000)	0.0027 (0.0002)	
Lactic	0.0766 (0.0031)	0.0198 (0.0008)	0.0005 (0.0001)	
Acetic	0.1800 (0.0011)	0.1002 (0.0026)	0.0025 (0.0001)	
L/A (1:1)	0.1833 (0.0054)	0.0900 (0.0035)	0.0024 (0.0002)	Expressed as lactic
	0.1223 (0.0036)	0.0600 (0.0024)	0.0015 (0.0002)	Expressed as acetic
L/A (2:1)	0.1519 (0.0044)	0.0675 (0.0038)	0.0012 (0.0001)	Expressed as lactic
	0.1012 (0.0029)	0.0450 (0.0025)	0.0008 (0.0001)	Expressed as acetic

[a] Mean.
[b] Standard deviation.

ficult to evaluate. At pH 6.5, the acid intensities were none to slight; thus, the most consistent agreement was found due to similar, weak to nonexistent flavor profiles.

Titratable acidity

Titratable acidity (TA) measurements (Table 7) showed, as expected, acetic acid at pH 3.5 had the highest TA. It has the highest pKa (4.75), whereas lactic acid had the lowest TA at all pH levels and has the lowest pKa (3.08). Sourness appeared related to pKa of the acids. Those with higher pKa were more sour, which confirmed research by Straub (1992). However, the farther the pH was from the pKa, the less the degree of difference that could be detected. TA has been hypothesized to be related to sourness (Harvey, 1920; Plane et al., 1980; Noble et al., 1986), while other researchers have reported that the two were slightly or not at all related (Rubico, 1992; Straub, 1992).

CONCLUSIONS

VARIOUS FOOD ACIDS at a given pH level have significantly different flavor profiles. The flavor profile of a given acid changes with pH level. Awareness of such differences in flavor of acidulants can be used to formulate acidified food products with specific sensory characteristics. Acids at pH 4.5 vary the most in sourness and OI. However, at pH 6.5 little difference exists between acid flavor profiles.

REFERENCES

ASTM. 1993. Standard termino ogy relating to sensory evaluation of material and products. In *Annual Book of ASTM Standards*, Section 15, General Products, Chemical Specialties, and End Use Products, Vol. 15.07, designation: E 253-93a. American Society for Testing and Materials, Philadelphia, PA.

Andres, C. 1985. Acidulants. Flavor, preserve, texturize, and leaven foods. Food Process. 46(5): 52–54.

Arnold, M.H.M. 1975. *Acidulants for Foods and Beverages*. Food Trade Press, London.

Bartoshuk, L.M. 1978. History of taste research. Ch. 1, in *Handbook of Perception. Vol. VIA. Tasting and Smelling*, E.C. Carterette and M.P. Friedman (Ed.), p. 3–18. Academic Press, New York.

Bate-Smith, E.C. 1954. Astringency in foods. Food Proc. Pack. 23: 124–127.

Bate-Smith, E.C. 1973. Haemanalysis of tannins: the concept of relative astringency. Phytochemistry (Oxf). 12: 907–912.

CoSeteng, M.Y., McLellan, M.R., and Downing, D.L. 1989. Influence of titratable acidity and pH on intensity of sourness of citric, malic, tartaric, lactic and acetic acids solutions and on the overall acceptability of imitation apple juice. Can. Inst. Food Sci. Technol. J. 22(1): 46–51.

Dijksterhuis, G.B. and van Buuren, S. 1989. *PROCRUSTES-PC v2.0 Manual*. OP&P, Utrecht, Holland.

Doores, S. 1990. pH control agents and acidulants. In *Food Additives*, A.L. Branen, P.M. Davidson, and S. Salminen (Ed.), p. 477–510. Marcel Dekker, Inc., New York.

Fabian, F.W. and Wadsworth, C.K. 1939. Experimental work on lactic acid in preserving pickles and pickle products. III. Use of lactic acid in pickles and related products. Food Res. 4: 521–529.

Gardner, W.H. 1972. Acidulants in food processing. Ch. 1, in *Handbook of Food Additives*, T.E. Furia (Ed.), 2nd ed., p. 225–270. CRC Press, Cleveland, OH.

Harvey, R.B. 1920. The relation between the total acidity, the concentration of the hydrogen ion and the taste of acid solutions. J. Amer. Chem. Soc. 42: 712–714.

Lawless, H.T., Corrigan, C.J., and Lee, C.B. 1994. Interactions of astringent substances. Chem. Senses. 19: 141–154.

Noble, A.C., Philbrick, K.C., and Boulton, R.B. 1986. Comparison of sourness of organic acid anions at equal pH and equal titratable acidity. J. Sens. Stud. 1(1): 1–7.

Pangborn, R.M. 1963. Relative taste intensities of selected sugars and organic acids. J. Food Sci. 28: 725–733.

Plane, R.A., Mattick, L.R., and Weirs, L.D. 1980. An acidity index for the taste of wines. Amer. J. Enol. Vitic. 31: 265–268.

Pszczola, D.E. 1988. Miles adds malic and fumaric acids. Food Technol. 42(6): 49.

Rubico, S.M. 1993. Perceptual characteristics of selected acidulants by different sensory and multivariate methods. Ph.D. thesis, Oregon State Univ., Corvallis.

Rubico, S.M. and McDaniel, M.R. 1992. Sensory evaluation of acids by free-choice profiling. Chem Senses. 17(3): 273–289.

SAS Institute, Inc. 1987. *SAS/STAT Guide for Personal Computers. Version 6*. SAS Institute Inc., Cary, NC.

Straub, A.M. 1992. Power funct on determination of sourness and astringency and time-intensity measurements of sourness and astringency for selected acids. MSc Thesis, Oregon State University, Corvallis.

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Starch Related Changes in Stored Soft Sorghum Porridge

C.T. MOKHORO and D.S. JACKSON

ABSTRACT

Soft sorghum (*Sorghum bicolor* (L.) Moench) unfermented porridge was prepared by cooking pearled sorghum flour (6% solids in water) in a microwave oven. Fermented porridge was prepared by mixing with *Lactobacillus delbruekii* for 12 hr at 48°C, followed by cooking. Porridge was aged (0–12 hr) at 25°C or 7.5°C. Starch polymer solubility was determined by high performance size exclusion chromatography (HPSEC) and rheology followed with a rotational viscometer. During aging, solubilized and gelled amylose and amylopectin retrogradation rates were higher in porridge stored at 7.5 than at 25°C. Amylose polymer solubility decreased with storage, while solubilized amylopectin remained in solution. Fermented porridges were lighter in color and their initial consistency indexes increased faster. HPSEC of soluble polymers was more sensitive than enzymatic starch digestion.

Key Words: sorghum, porridge, starch, fermentation

INTRODUCTION

SORGHUM (*Sorghum bicolor* (L.) Moench) is an important cereal grain in the world, in terms of the quantities produced. Human consumption is limited to semi-arid areas of Africa, Asia, and Latin America where it is an important agricultural product due to drought tolerance. Continuing consumption of sorghum is usually restricted to low-income consumers or to times when no other cereal grain is available. Understanding and improving quality characteristics of sorghum as human food may help save resources by reducing importation of other grains.

Sorghum is most often consumed in the form of porridge (Hoseney et al., 1987), (gruels) made from water and flour. A soft porridge is a thin, easily flowing product with low solids, while a thick porridge is a stiff product with higher solids and can be molded and eaten by hand (Bello et al., 1990). Unfermented porridge is generally prepared for immediate consumption as quality quickly deteriorates with time and it is susceptible to microbial spoilage. Fermented porridge has attracted attention both due to its microbial stability and improved nutritional value (Au and Fields, 1981; Kazanas and Fields, 1981; Adeyemi and Beckley, 1986) as well as improved flavor and palatability.

Fermented porridge generally involves natural fermentation. Commercially available fermented products are commonly produced with formulations based on traditional processes. One such product is *mahleu* or *mageu*, produced commercially in the Republic of South Africa, which uses *Lactobacillus delbruekii* as fermenting organism (Holzapfel, 1989).

Porridges may be kept for varying times before they are consumed. A better understanding of their properties could improve the potential for commercial processing and increased utilization of sorghum products for human consumption. As in other cereal grains, starch is the main constituent of sorghum, and it is important in determining functional properties of sorghum products. Two of the most important criteria affecting quality of sorghum porridge are color and consistency; a light colored and free flowing product is preferred (Hulse et al., 1980). Although usually prepared for immediate use (Obilana, 1982), soft porridge may be stored as in the case of baby foods, so as to easily provide nutrients throughout the day. Since sorghum has a substantial starch content, porridge were expected to exhibit properties characteristic of starch pastes, such as increased viscosity and reduced paste clarity over time.

Although some quality characteristics of thick sorghum porridge are understood (Akingbala et al., 1981; Cagampang et al., 1982; Rooney and Murty, 1982; Cagampang and Kirleis, 1984; Bello et al., 1990), the quality of soft (or thin) sorghum porridge has been mostly deduced from those studies or from other cereals. Information is particularly lacking on starch related changes during storage of thin porridges. Such starch changes would likely be more subtle and difficult to measure than for thick porridges. Our objective was to develop a laboratory process for making soft sorghum porridge and to study rheological and starch solubility properties of fermented and unfermented products during storage.

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MATERIALS & METHODS

Cleaning, pearling and milling

White sorghum (*Sorghum bicolor* (L.) Moench, Northrup King 714, 1992 season) was obtained from a farm in Southeastern Nebraska. The sorghum was cleaned by sieving through a 2.8 mm screen (U.S. Standard No. 7 sieve, Tyler Inc., Ohio) on a strand shaker (Strand Shaker Co., Minneapolis, MN, serial no. SS 151) for one min. Large debris were removed by hand. Cleaned sorghum was accurately weighed in 10 kg batches and pearled for 1.5 min in a custom-made dehuller. The 1.5 min pearling time was the optimum time for removal of most of the bran while minimizing grain breakage. The sorghum was again sieved through a No. 7 sieve on the strand shaker for 3 min after which the final weight was determined. To indicate how much material was lost on pearling, the pearling index of the sorghum was calculated by the difference in weight as percentage of original sorghum. The pearled sorghum was then milled on a Wiley Mill No. 1 (Arthur H Thomas Co.) connected in series to a Jackobson pulverator (Jackobson Inc., Model No. 66B). Flour was sieved to pass through a 850 mm screen before use. The pearled sorghum flour was kept frozen in a sealed container at −20°C until used. Standard AOAC (1990) methods were used to determine fat (922.06), moisture (925.10) and ash (923.03). Protein was determined by a modification of AOAC method 981.10 (1990) as follows: 1g sample was heated in 20 mL acid at 400°C. Total starch was determined using format 2 of the Megazyme Total Starch Assay Kit (Megazyme, Inc., Warriewood, AUSTRALIA). Nonstarch carbohydrate was calculated by difference.

Porridge preparation

Unfermented porridge was prepared by adding flour (270g) to deionized water (4.5 L) and mixing thoroughly. The slurry was cooked in a microwave (Quasar Model no. MQ8850TW, 700 W) set on high power. The slurry was stirred (six times clockwise and six times counter clockwise) every 5 min for the first 20 min and every 2 min thereafter until boiling began. Heating was continued for an additional 10 min, after which the porridge was stirred again. Microwave energy was preferred over conventional heating for cooking the relatively large laboratory samples, since microwave energy can be increased or stopped "instantaneously." Microwave heating of laboratory samples allowed for greater temperature and cooking control as compared with cooking in a water bath (data not shown). Portions (300 mL) of the porridge were dispensed in 400 mL beakers and covered with aluminum foil. Portions were stored in a controlled temperature cabinet at either 7.5 or 25°C for 0–12 hr.

To prepare a starter culture for fermented porridge, a slurry of 50 g sorghum flour and 200 mL deionized water was inoculated with *Lactobacillus delbruekii* cells and incubated at 48°C. Every 24 hr a new starter was similarly prepared by adding an inoculum (10 mL) from each previous fermentation; five consecutive inoculations were carried out in this manner prior to using any starter for porridge preparation. Standard growth curves of the bacterial cells, to insure reproducibility and measure bacterial cell numbers, were prepared by plating serially diluted inoculum on de Man, Rogosa and Sharpe (MRS) broth mixed with 1.5% agar at 32°C every 3 hr over the 24 hr period. Fermented porridge was prepared by inoculating a slurry of 270 g flour in 4470 mL distilled water with 30 mL of an inoculum from a 12 hr old culture of *Lactobacillus delbruekii*, incubating for 24 hr at 48°C, and cooking as for unfermented porridge, except that stirring was initially at 3 min intervals for the first 12 min. Measurements of pH were made on fermented slurries just before cooking and on freshly cooked porridges.

Rheological measurements

A Brookfield Synchro-Lectric viscometer (Brookfield Engineering Laboratories, Stoughton, MA; LVT Model, Serial no. 29355) was used for all rheological determinations. Porridge aged from 0-12 hr was transferred to the Griffin beaker provided with viscometer and was stirred thoroughly. Porridge temperature was recorded and the beaker was placed in a water bath set at the same temperature as the porridge. Torque readings were recorded at different speeds using spindles No. 1, 2 and 3 depending on consistency of the sorghum, to give at least four readings within the instrument scale reading. Thus, spindle No. 1 was used initially and the successively smaller spindles (No. 2 and 3) were used as consistency increased. Readings were taken 20 sec after the instrument was turned on. Flow behavior index of each porridge was determined as the slope of the spindle speeds vs corresponding torque readings. Shear stress was calculated using equation 1, and shear rate was calculated using equation 2.

$$\tau = \frac{A}{2\pi R^2} \quad (1)$$

where τ = shear stress; A = torque measured; L = submerged length of spindle; and R = radius of the spindle.

$$\gamma = \frac{4\pi N}{n} \quad (2)$$

where γ = shear rate; N = rotational speed; and n = flow behavior index.

By plotting Log shear rate vs Log shear stress, consistency index was determined as the intercept at the point where shear rate = 1. Sample temperatures prior to and after rheological measurements were recorded to detect if large (>2°C) temperature fluctuations had occurred.

Starch enzyme susceptibility

A portion of each porridge sample was transferred to a 15 mL cryogenic vial (Nalge Company, Rochester, NY) filled to half capacity. The vial was then dipped in liquid nitrogen for 40 sec and the frozen sample freeze dried. Samples were stored at −20°C when not freeze-dried immediately. After regrinding the freeze-dried sample using a mortar and pestle to pass through a 600 mesh screen, starch enzyme susceptibility was determined by digesting the sample (200 mg) in 10 mL 3-[N]propane-sulfonic acid (MOPS)(Sigma, St. Louis, MO) buffer at pH 7 with 0.5 mL of thermostable α-amylase [EC 3.2.1.1, 40 I.U./mL] for 20 min at 25°C. The reaction was stopped by heating the sample to boiling (20 sec) in a microwave oven. Contents of the test tube were quantitatively transferred to a 100 mL volumetric flask and distilled water added to the mark; samples were next filtered through Whatman No. 1 filter paper. An aliquot (0.2 mL) of the filtrate was transferred to a test tube and 0.5 mL maltase (EC 3.2.1.20, Sigma, 4 I.U./mL) plus 2.5 mL of a buffered glucose oxidase/peroxidase/4-aminoantipyrine (GOPOD) reagent were added. The mixture was incubated at 25°C for 1 hr and absorbence measured at 510 nm using a spectrophotometer (Perkin-Elmer Lambda 3B, Model No. C618-0437). Digested starch was calculated using the Megazyme (Warriewood, AUSTRALIA) procedure (Anonymous. 1992). To determine total starch, all samples in each replicate were mixed together and total starch determined by Format 2 of the Megazyme procedure (Anonymous 1992). Digested starch was expressed as % of total starch to obtain starch digestion index (SDI), based on the procedure by Englyst, Kingsman and Cummings (1992).

Starch polymer solubility

Each aged porridge sample was centrifuged in a 50 mL tube for 10 min at 3000 × g. Two mL of supernatant was shaken with 250 mg (125 mg/mL) of mixed bed ion exchange resin (AG 501-X and Bio-Rad MSZ 501 (D), Bio-Rad, Hercules, CA) at room temperature (≈23°C) for 30 min. The proper level of resin was determined by following the procedure described by Zhang and Jackson (1992). Samples were filtered through a 0.45 mm nylon filter and 25 μL aliquots were injected into a High Performance Size Exclusion Chromatography (HPSEC) system (Zhang and Jackson, 1992). Previous data (Jackson et al., 1989) was used to identify amylose and amylopectin peaks. The relative amount of starch in solution was determined by calculating the area under starch curves; while absolute solubility was estimated as described by Jackson (1988). The apparent solubility of each starch component (amylose or amylopectin) was also calculated as percentage of total starch solubility to determine if properties could better be described as relative amounts of solubilized amylose and amylopectin than as total solubilized starch.

Experimental design and analysis

For each parameter, 3 replicate porridge samples were prepared and analysis of variance (ANOVA) performed using the Statistical Analysis System (SAS Institute, Inc. 1989). Regression analysis was performed using SAS to determine relationships between time and all measured variables using the model defined by Eq. (3).

$$\gamma = B_0 + B_1X + B_2X^2 + \varepsilon \quad (3)$$

where γ = dependent variable; β = parameters which specify nature of the relationship; χ = time (the independent variable in this case); and ε = random error.

Means and coefficients of variation (CV) were also determined using SAS. Statistical significance was established at $P \leq 0.05$.

RESULTS & DISCUSSION

Pearled sorghum and flour porridge

The pearling index of sorghum was 15%. The average proximate composition of sorghum flour was 11.7% moisture (CV=0.34%), 4.3% fat (CV=4.5%), 7.3% protein (N × 6.25, CV=0.75%), 1.4% ash (CV=5.6%), 74% starch (CV=2.2%) and 1.3% nonstarch carbohydrates (by subtraction). Where sorghum is used for human consumption, wide variations of both unfermented and fermented soft porridges exist, especially with regard to methods of preparation and porridge consistency. Although porridge preparation was based on traditional processes, it was mainly prepared to our personal specifications. A six percent (6%) solid content was found most acceptable.

Unfermented porridge

Immediately after cooking (0 hr) the freshly cooked porridge was a thin flowable paste. It rapidly changed in consistency when stored at both 25 and 7.5°C, so that within 1 hr it was no longer free-flowing. This was indicative of gel formation. Stirring the porridge easily restored flow properties in less aged samples, suggesting the gel structure had not been rigidly formed and was thixotropic. The porridge initially appeared gray but darkened over time, particularly when stored at 7.5°C.

Changes in relative aqueous solubility of amylose and amylopectin molecules over time were measured by HPSEC and compared (Fig. 1). The porridges were relatively concentrated starch systems, and our aqueous HPSEC (without sonication) only measured those polymers dispersed in solution and not partially aggregated or entangled. Thus our total estimated starch polymer solubility for the unfermented porridges was only 6% immediately after cooking. The average temperature profiles for both stored porridges were compared (Fig. 2). For that stored at 7.5°C, apparent amylopectin solubility decreased with time, indicating that a small amount of soluble amylopectin aggregated from solution. Amylopectin solubility was not described by a regression model, with time for unfermented porridges stored at 25°C, indicating the relative stability of these polymers at that temperature (Table 1, Fig. 1). Solubilized amylose, however,

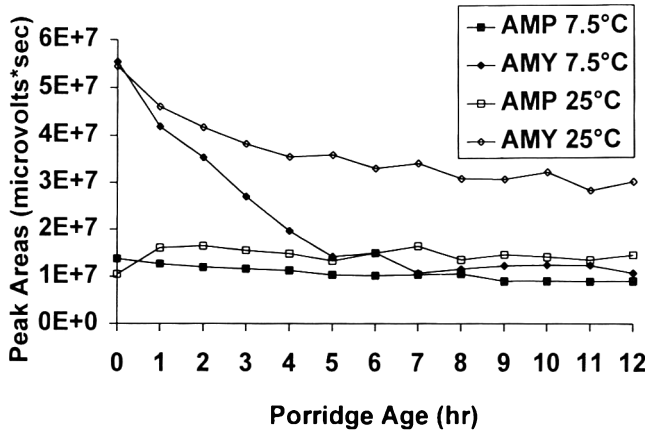

Fig. 1—Changes in apparent solubility of amylose and amylopectin in soft unfermented sorghum porridges. AMY = amylose; AMP = amylopectin; 7.5°C and 25°C refer to storage temperature.

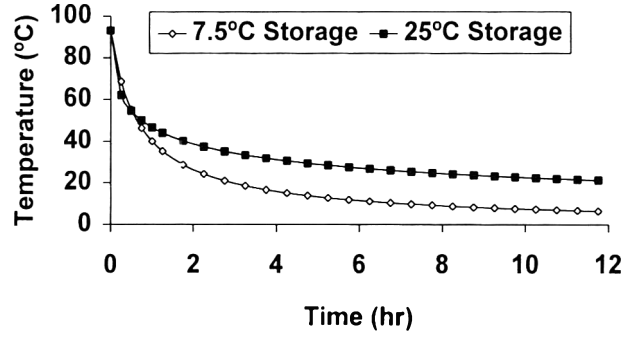

Fig. 2—Mean changes in porridge temperatures upon storage at 7.5 and 25°C for soft fermented and unfermented sorghum porridge.

decreased more at both temperatures over time; soluble amylose significantly modeled with time. HPSEC starch solubility profiles of unfermented porridge were compared (Fig. 3). They showed rapid decline in size of amylose peaks relative to amylopectin, with a more rapid decline in amylose peak size for porridges stored at 7.5 than at 25°C. In the porridge stored at 7.5°C, the amount of soluble amylopectin remained about the same, while the amylose decreased. Amylose was precipitating from solution so that its relative concentration vs amylopectin was decreasing. Starch digestion index of porridge stored at 7.5 and 25°C averaged 22 and 23%. Digestion index was not described using a regression model with time, nor were values at 7.5 and 25°C statistically different ($\alpha = 0.05$).

As starch molecules aggregate, they impart changes in properties of starch paste which affect rheology. A regression model for the stored porridges described flow behavior index (n) decreasing with increasing time (storage) at both temperatures (Table 1, Fig. 4). For porridge stored at 25°C, this apparent decrease was only due to the large difference between means at time 0 hr and the means at other times. The flow behavior index (n) changed from a nearly Newtonian to pseudoplastic behavior in 1 hr of storage (Fig. 4). According to Toledo (1991), n is not influenced by temperature unless accompanied by chemical changes. We expected that a change of temperature from over 90°C (time 0 hr) to about 40°C (porridge aged for 1 hr at 25°C) would be accompanied by retrogradation, which is a physicochemical change in starch structure. The significant differences between flow behavior index means at 7.5°C, therefore, suggested that more pronounced changes occurred at that temperature resulting in notable chemical changes than at 25°C. This was consistent with the gelation and retrogradation behavior of starch. Since all porridges were cooled (about 1 hr at room temperature), prior to consumption, we could conclude that, in the porridge stored at 25°C, n did not change significantly during the time in which it would normally be consumed (1–12 hr).

The regression model for consistency index (k) and time was significant (Table 1); k increased with time for both storage

Table 1—Coefficients of determination and probability values for regression models of dependent variables

Porridge type	Temp	Dependent variable	R^2 of regression	P-Values[a] for time	P-Values for time^2
Unfermented	25°C	Amylopectin	0.030	0.3196	0.3000
		Amylose	0.774	**0.0001**	**0.0001**
		Flow Behavior Index (n)	0.500	**0.0001**	**0.0001**
		Consistency Index (k)	0.363	**0.0002**	**0.0008**
		Starch Digestion Index	0.017	0.8876	0.9441
	7.5°C	Amylopectin	0.526	**0.0053**	0.1794
		Amylose	0.935	**0.0001**	**0.0001**
		Flow Behavior Index (n)	0.659	**0.0001**	**0.0004**
		Consistency Index (k)	0.929	**0.0001**	**0.0001**
		Starch Digestion Index	0.233	0.7955	0.2824
Fermented	25°C	Amylopectin	0.291	**0.0444**	0.2584
		Amylose	0.583	**0.0001**	**0.0054**
		Flow Behavior Index (n)	0.358	**0.0004**	**0.0031**
		Consistency Index (k)	0.770	**0.0001**	0.0009
		Starch Digestion Index	0.094	0.1682	0.0980
	7.5°C	Amylopectin	0.128	0.3679	0.1585
		Amylose	0.872	**0.0001**	**0.0001**
		Flow Behavior Index (n)	0.325	**0.0007**	**0.0038**
		Consistency Index (k)	0.811	**0.0001**	**0.0001**
		Starch Digestion Index	0.175	0.2634	0.6739

[a] Pr > T for testing H_0: parameter = 0; significant parameter estimates are bolded (0.05 level).

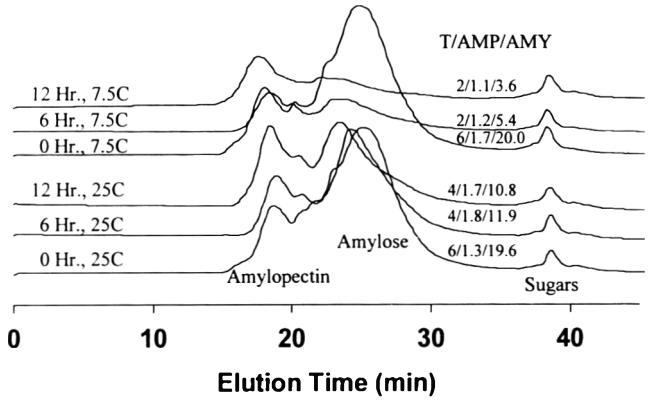

Fig. 3—Representative high performance size exclusion chromatograms of starch from soft unfermented sorghum porridge stored at 7.5 and 25°C. T = Estimated percent total starch polymer solubility; AMP & AMY = Estimated percent amylopectin (AMP) and amylose (AMY) polymer solubility given sorghum with a 25% amylose, 75% amylopectin content.

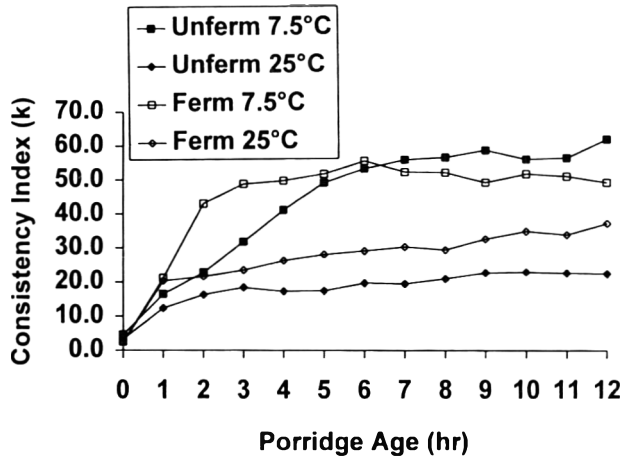

Fig. 5—Changes in consistency index (k) during storage of soft porridge. Unferm = Unfermented porride; Ferm = Fermented porridge; 7.5°C and 25°C refer to storage temperatures.

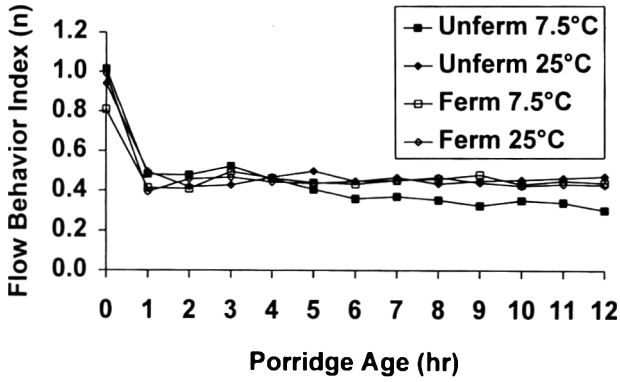

Fig. 4—Flow behavior indices (n) of soft unfermented and fermented sorghum porridge during storage. Unferm = Unfermented porridge; Ferm = Fermented porridge; 7.5°C and 25°C refer to storage temperatures.

temperatures. This indicated that a gel was formed in both porridges resulting in increased resistance to flow. Porridges kept at 7.5°C attained higher average k values than those stored at 25°C (Fig. 5).

These observations are consistent with the concept of gelatinized starch aggregation. Gelatinized amylose has a tendency to aggregate, even at room temperature, with a resultant decrease in solubility (Collison, 1968; Miles et al., 1985a; Ring, 1987). Using dilatometry, turbidity, and rheological measurements, Miles et al. (1985a,b) established that aggregation of amylose from solution occurred in minutes. Gelatinized amylopectin is more stable in solution than amylose; Ring et al. (1987) stored waxy maize amylopectin solutions at 1-20°C and found that polymer aggregation was more evident at lower temperatures. In porridges stored at 7.5°C, temperature decline slowed markedly after about 6–7 hr storage (Fig. 2). The rapid drop in apparent amylose solubility during that time could be explained in terms of combined changes in time and temperature. Our findings indicate that the behavior of starch in unfermented porridge was consistent with its behavior in a relatively homogeneous system (purified starch).

Fermented porridge

The microbial population in the initial starter culture increased to a maximum (10^8 cells/mL) after about 12 hr and then declined steadily so that there was about one log reduction over the next 6 hours. After several repeated inoculations, the population increased to a maximum in a relatively shorter time (9 hr) and the high counts remained stable from 9–15 hr at 10^8 cells/mL, probably indicating adaptation to the substrate. Almost all colonies had the same color and changed during growth from a milky appearance to an orange yellow color, suggesting contamination was minimal. All inoculations of fermented porridges took place using 12 hr old starter culture.

Immediately after cooking (0 hr), the freshly cooked fermented porridge was a creamy-white, easily poured fluid. Porridges had pH values averaging 3.5 (CV=4.8%). Soon after removal of heat, the porridge started to lose its fluidity so that differences in consistency between 0 hr and 1 hr were apparent. The cream-white appearance vs the gray color of unfermented porridges, was likely caused by a fermentation induced bleaching effect consistent with previously reported observations. Reichert (1979) found that whole millet flour pastes prepared at different pH ranges were creamy, gray and yellow at low, medium and high pH respectively. He suggested that acid treatment dissociated or modified some of the millet pigments. We noted no change in color over time in all fermented porridges.

Observed changes in fermented porridges were more difficult to interpret because of formation of acid during fermentation and resulting lower pH during storage. In addition, starch from unstored porridge might also have been affected, to a limited extent, by bacterial α- amylase activity prior to inactivation during cooking. Possibly two opposing events were taking place during porridge storage; the aggregation (effectively "polymerization") of polymer molecules as they retrograde, and hydrolysis of polymer molecules by the acid (depolymerization). Overall changes would then depend on the relative rates of these processes.

Amylose solubility of fermented porridge was significantly modeled (by regression) with time (Table 1) for porridge stored at 25 and 7.5°C; amylose decreased with time. Although a significant model was calculated for amylopectin solubility with time for fermented porridge stored at 25°C (Table 1), there were generally, no differences between means for different times from 1 hr to 12 hr. Likely starch solubility increased initially due to acid hydrolysis, but further increases were offset by molecular aggregation during storage. Changes in apparent solubility of amylose and amylopectin were compared in fermented porridges (Fig. 6). Total estimated polymer solubility (8% immediately after cooking) was slightly greater than for unfermented porridge. Starch solubility, by HPSEC, also increased when corn starch was heated for 30 min in pH 3.5 solution of lactic acid and water (data not shown). Fermented porridge showed the appearance of an intermediate fraction between the amylopectin and amylose peaks indicating amylopectin was hydrolyzed by the acid (Fig. 7). Results were similar to those when pure starch systems were excessively sonicated (Jackson et al., 1988). They

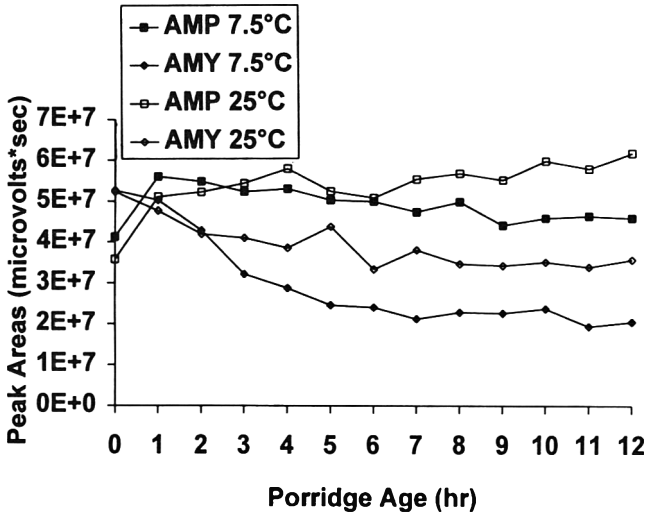

Fig. 6—Changes in apparent solubility of amylose and amylopectin in soft fermented sorghum porridge. AMY = amylose; AMP = amylopectin; 7.5°C and 25°C refer to storage temperatures.

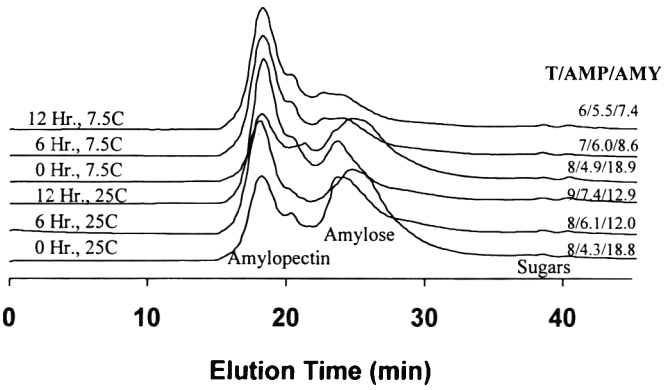

Fig. 7—Representative high performance size exclusion chromatograms of starch from soft fermented sorghum porridge stored at 7.5 and 25°C. T = Estimated percent total starch polymer solubility; AMP & AMY = Estimated percent amylopectin (AMP) and amylose (AMY) polymer solubility given sorghum with a 25% amylose, 75% amylopectin content.

reported that moderate sonication increased starch solubility by disrupting granule integrity, but long-term solubility decreased when starch was excessively sonicated creating polymers sizes and more linear structures conducive to aggregation (similar in molecular weights and/or branching characteristics to amylose). Researchers from our laboratory have also reported that acid depolymerized starches had increased retrogradation rates (Zhang and Jackson, 1992). In a similar fashion, acid disrupts granules in the porridge and increases solubility, while at the same time molecular association is increased due to hydrolysis of starch. Two opposing forces are in effect. Starch digestion index of fermented porridge stored at 7.5 and 25°C averaged 46 and 51%, respectively. As with unfermented porridge, digestion index was not successfully described using a regression model with time, but values at 7.5 and 25°C were statistically different ($\alpha = 0.05$). The digestion indices were not as sensitive to starch changes as HPSEC analysis.

The flow behavior index for fermented porridge stored at both temperatures was less than unity, indicating a pseudoplastic flow behavior (Fig. 4). An overall decrease in the flow behavior index occurred probably for the same reason as in unfermented porridge. Again, the means from time 1 hr to 12 hr were not significantly different within or between porridges suggesting that storage temperature did not significantly affect n (Toledo, 1991). Sopade and Kassum (1992) found no change in n in fermented porridge prepared from sorghum, millet or maize, measured at 10°C to 70°C. A very good regression model described the consistency index and time; consistency index increased with time (Table 1, Fig. 5). Since both k and n relate to ease with which a fluid flows, these observations indicate that aggregation of molecules was substantial and resulted in increased resistance to flow.

Overall changes

Obvious differences occurred between fermented and unfermented porridge, some of which could be fully explained by starch properties. HPSEC data showed lower soluble amylose to amylopectin ratios in freshly cooked fermented porridges. This may be due to acidic hydrolysis of weak amylopectin-amylopectin associations or entanglements in dispersed starch solutions which interfere with HPSEC analysis. The same HPSEC also showed a low concentration of very low molecular carbohydrates (sugars) in fermented porridges (Fig. 3 vs Fig. 7). This would suggest that. following hydrolysis into glucose, these carbohydrates were used during fermentation.

Fermented porridges showed faster initial increases in consistency index (k) compared to unfermented porridges at the same storage temperature. This was probably due to hydrolysis of starch in fermented porridges. Whistler and Johnson (1948) have shown that as amylose was hydrolyzed, its rate of retrogradation increased to a certain point beyond which it decreased again. Similarly, Zhang and Jackson (1992) reported that the initial rate of retrogradation increased when starch was hydrolyzed. They attributed this increase to increased mobility of hydrolyzed amylopectin. Fermented porridges had higher starch digestion index values also, perhaps because acid increased susceptibility to enzyme hydrolysis.

CONCLUSIONS

CHANGES OCCURRED IN PORRIDGE characteristics during storage. The degree of starch retrogradation was higher in aged porridge stored at lower temperatures compared to freshly prepared samples or those stored at higher temperatures. Apparent solubility of amylose decreased more at lower temperature due to reassociation and precipitation of amylose from solution. Amylopectin was relatively more stable in solution. Consistency indexes also increased due to cross-link network formation on aggregation of molecules. Fermented porridges had lighter color probably due to bleaching effects of acid during fermentation. Acid hydrolysis after starch gelatinization (cooking) resulted in higher initial retrogradation in fermented porridges. HPSEC was sensitive to changes in porridge over time and paralleled rheological changes. Enzyme susceptibility was higher for fermented porridges but was less sensitive to changes than was HPSEC analysis. Starch has a strong influence on storage properties of sorghum porridge consistent with observations in pure starch systems. Therefore techniques to study pure starch systems could be valid for thin sorghum porridge. The preparation methods we developed enabled porridges to be prepared with reproducibility adequate for statistical determination of significant changes in starch characteristics which could be easily monitored.

REFERENCES

Adeyemi, I.A. and Beckley, O. 1986. Effects of period of maize fermentation and souring on chemical properties and amylograph pasting viscosity of ogi. J. Cereal Sci. 4: 353.
Akingbala, J.O., Gomez, M.H., Rooney, L.W., and Sweat, V.E. 1988. Thermal properties of sorghum starch. Starch/Starke 40(10): 375.
Anon. 1992. *Total Starch Assay Procedure*. Megazyme Inc., Warriewood, Australia.
AOAC. 1990. *Official Methods of Analysis*. Assoc. Official Analytical Chemists, Arlington, VA.
Au, P.M. and Fields, M.L. 1981. Nutritive value of fermented sorghum. J. Food Sci. 46: 562.
Bello, A.B., Rooney, L.W., and Waniska, R.D. 1990. Factors affecting quality of sorghum to, a thick porridge. Cereal Chem. 67(1): 20.

Bourne, M.C. 1982. *Food Texture and Viscosity: Concept and Measurement.* Academic Press, New York.
Cagampang, G.B. and Kerleis, A.W. 1984. Relationship of sorghum grain hardness to selected physical and chemical measurement of grain quality. Cereal Chem. 61(2): 100.
Cagampang, G.B., Griffith, J.E., and Kerleis, A.W. 1982. Note: Modified adhesion test for measuring stickiness of sorghum porridges. Cereal Chem. 59: 234.
Collison, R. 1968. Starch retrogradation. *Starch and its Derivatives,* 4th ed., J.A. Radley (Ed.), p. 294. Chapman and Hall, London, UK.
Englyst, H.N., Kingman, S.M., and Cummings, J.H. 1992. Classification and measurement on nutritionally important starch fractions. Eur. J. Clin. Nutr. 46 (Suppl. 2): S33–S50.
Holzapfel, W.H. 1989. Industrialization of mageu fermentation in South Africa. In *Industrialization of Indigenous Fermented Foods,* K.H. Steinkraus (Ed.), p. 285. Marcel Dekker, New York.
Hoseney, R.C., Andrews, D.J., and Clark, H. 1987. Sorghum and millet. In *Nutritional Quality of Cereal Grains: Genetic and Agronomic Improvement,* R.A. Olson and K.J. Frey (Ed.), p. 397. American Society Agronomy.
Hulse, J.H., Laing, E.M., and Pearson, O.E. 1980. *Sorghum and the Millets: Their Composition and Nutritional Value.* Academic Press, New York.
Jackson, D.S. 1988. Characterization of Starch by High-Performance Liquid Chromatography-Size Exclusion Chromatography (HPLC-SEC). Ph.D. thesis, Texas A&M Univ., College Station, TX.
Jackson, D.S., Choto-Owen, C., Waniska, R.D., and Rooney, L.W. 1988. Characterization of starch cooked in alkali by aqueous high-performance size exclusion chromatography. Cereal Chem. 65(6): 493.
Jackson, D.S., Waniska, R.D., and Rooney, L.W. 1989. Differential water solubility of corn and sorghum starches as characterized by high-performance size exclusion chromatography. Cereal Chem. 66(3): 228.
Kazanas, N. and Fields, M.L. 1981. Nutritional improvement of sorghum by fermentation. J. Food Sci. 46: 819.
Miles, M.J., Morris, V.J., and Ring, S.G. 1985a. Gelation of amylose. Carbohydr. Res. 135: 257.
Miles, M.J., Morris, V.J., Orford, P.D., and Ring, S.G. 1985b. The roles of amylose and amylopectin in gelation and retrogradation of starch. Carbohydr. Res. 135: 271.
Obilana, A.T. 1982. Traditional sorghum foods in Nigeria: their preparation and quality parameters. In *Proceedings of the International Symposium on Sorghum Grain Quality,* L.W. Rooney and D.S. Murty (Ed.), p. 45. ICRISAT, Patancheru, India.
Reichert, R.D. 1979. The pH-sensitive pigments in pearl millet. Cereal Chem. 56(4): 291–294.
Ring, S.G., Colonna, P., Tanson, K., Kalicevsky, M.T., Miles, M., Morris, V.J., and Orford, P.D. 1987. The gelation and crystallization of amylopectin. Carbohydr. Res. 162: 277.
Ring, S.G. 1987. Molecular interaction in aqueous solutions of starch polysaccharides: a review. Food Hydrocolloids 1: 449.
Rooney, L.W. and Murty D.S. (Ed.), 1982. International symposium on sorghum grain quality. ICRISAT, Patancheru, India.
SAS Institute, Inc. 1989. *Proprietary Software Release 6.08.* SAS Institute, Inc. Cary, NC.
Sopade, P.A. and Kassum, A.L. 1992. Rheological characterization of akamu, a semi-liquid food from maize, millet and sorghum. J. of Cereal Sci. 15: 193–202.
Toledo, R.T. 1991. *Fundamentals of Food Process Engineering,* 2nd ed. Van Nostrand Reinhold, New York.
Whistler, R.L. and Johnson, C. 1948. Effect of acid hydrolysis on the retrogradation of amylose. Cereal Chem. 25: 424.
Zhang, W. and Jackson, D.S. 1992. Retrogradation behavior of wheat starch gels with differing molecular profiles. J. Food Sci. 57: 1428.
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PROCESSING AND STORAGE OF HUMAN MILK. . .From page 377

philized milk stored for 4 mo and infused both at slow and rapid rates. In general, the concentration of protein in all infusates of both frozen and lyophilized milk samples was stable. Therefore, results of frozen storage of ultrasonicated milk for both 1 and 4 mo showed excellent recovery of protein. Lyophilization and subsequent storage of ultrasonicated milk resulted in only very slight fluctuations in protein recovery towards the end of slow infusion. Protein denaturation in fat globule membrane during lyophilization may account for this. The cumulative recovery of protein was 99% for both frozen and lyophilized samples infused at a slow rate after storage of 4 mo. In the rapidly infused milk, the recovery of protein was also essentially complete.

CONCLUSIONS

IF HUMAN MILK must be stored in the lyophilized form, it should be ultrasonicated just before use (i.e., after reconstitution), rather than before lyophilization, to give better fat recovery. If milk is to be stored frozen and not lyophilized, then ultrasonicating it before storage is adequate to provide efficient recovery of both fat and protein. In order to initiate the use of ultrasonicated human milk in feeding ill and premature babies, future research should include feeding trials with ultrasonicated breast milk giving prime emphasis to weight gain.

REFERENCES

Alemi, B., Hamosh, M., Svanlon, J.W., Salzmann, C., and Hamosh, P. 1981. Fat digestion in very low birth weight infants: Effect of addition of human milk to low-birth weight formula. Pediatrics 68: 484–488.
Garza, C., Schanler, R.J., Butte, N.F., and Motil, K.J. 1987. Special properties of human milk. Clin. Perinatol. 14: 11–32.
Greer, F.R., McCormick, A., and Loker, J. 1984. Changes in fat concentration of human milk during delivery by intermittent bolus and continuous mechanical pump infusion. J. Pediatr. 105: 745–747.
Martinez, F.E., Desai, I.D., Davidson, A.G.F., Nakai, S., and Radcliffe, A. 1987. Ultrasonic homogenization of expressed human milk to prevent nutrient loss during tube feeding. J. Ped. Gastroenterol. Nutr. 6: 593–597.
Nakai, S. and Chi, A.C. 1970. Spectrophotometric determination of protein and fat in milk simultaneously. J. Dairy Sci. 53: 276–278.
Stocks, R.J., Davies, D.P., Allen, F., and Sewell, D. 1985. Loss of breast milk nutrients during tube feeding. Arch. Disease Child. 60: 164–166.
Whitfield, M.F. 1982. Poor weight gain of the low birth weight infant fed naso-jejunally. Arch. Disease Child. 57: 597–601.
Ms received 7/4/94; revised 10/26/94; accepted 11/7/94.

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Some Antinutritional Factors in *Moringa peregrina* (Al-Yassar or Al-Ban) and Soybean Products

HASSAN A. AL-KAHTANI

ABSTRACT

Moringa peregrina and soybean defatted flours, protein concentrates, and isolates were assayed for trypsin (TIA) and α-amylase (AIA) inhibitor activities, phytic acid, tannin and chlorogenic acid contents, and *in vitro* protein digestibility (IVPD). TIA in *M. peregrina* defatted flour (MDF) was lower ($P < 0.05$) but more heat resistant than in soybean. AIA in MDF was lower than in soybean and inhibited pancreatic amylase more than bacterial amylase. Some *M.peregrina* products were higher in phytic acid but lower in chlorogenic acid than soybean. Tannin was low in all samples. IVPD was slightly lower for *M.peregrina* than for soybean.

Key Words: soybean, antinutrients, flour, protein concentrate, trypsin

INTRODUCTION

MORINGA PEREGRINA [Syns=*M.optera* Gaerton., *M.arabica* (Lam) Pers.] is one of about 10 xerophytic species of the family *Moringaceae* (FAO, 1988). Details on such plants, including English and French names, botanic characteristics, world distribution, chemical composition, and uses have been published (Migahid, 1978; Somali et al., 1984; FAO, 1988; Al-Yahya et al., 1990). The chemistry of crude oil (>54%) (Al-Kahtani, 1993), and the physical, chemical, and functional properties of *M.peregrina* proteins (Al-Kahtani and Abou-Arab, 1993) were also reported. The meal remaining after extraction of oil contains about 57% protein and could be a very important protein source if it is suitable for human consumption.

Nutritive quality or digestibility of a protein is affected by the presence of antinutritional factors, such as enzyme inhibitors, phenolic compounds, and phytate (Carter et al., 1972; Hsu et al., 1977; Powers and Whitaker, 1977; Granum, 1979; Erdman et al., 1980; Tan et al., 1983; Cinco et al., 1985; Kumar and Chauhan, 1993; Mameesh and Tomar, 1993; Ologhobo and Fetuga, 1984). No determination of antinutritional factors in *M.peregrina* has been made.

This work is a continuation of our earlier study on *M.peregrina* products (Al-Kahtani and Abou-Arab, 1993). The objective was to evaluate several possible antinutritional factors in *M peregrina* and compare them with soybean products, and to assess the *in vitro* protein digestibility of those products.

MATERIALS & METHODS

Materials

Seeds (kernels) of *M.peregrina* were obtained from Al-Ola region, Northwest Saudi Arabia. Soybeans (cv. Jupiter) were obtained from Agricultural Experiment Station, College of Agriculture, King Saud University, Riyadh, Saudi Arabia. Seeds were cleaned, hand-cracked, dehulled and pulverized with a Waring commercial blender (Sanyo Electric Co. Ltd., Japan) at speed 1 for 15 sec. Soybean seeds were milled to pass through 0.5 mm sieve using an Ultra-Centrifugal mill (Resh type ZMI, F., Kurt Retsch Gmb H & Co., Germany).

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Preparation of defatted flour, protein concentrate, and protein isolate

Full fat flours from M.peregrina and soybeans were prepared as described (Al-Kahtani and Abu-Arab, 1993). The defatted meals were air-dried for 24 hr at room temperature (≈25°C) and then ground into flour (180-μm particles). Protein concentrate was prepared from the defatted flour according to the methods of Mattil (1974). The method of Sosulski et al. (1978) was used for preparation of protein isolates. Protein concentrate and protein isolates were dried and ground into flour (180-μm particles).

Trypsin inhibitor activity assay

Trypsin inhibitor extracts were prepared as described by Roy and Bhat (1974). Four g powdered samples were extracted with 40 mL 0.05M sodium phosphate buffer, pH 7.0, and 40 mL distilled water. Samples were shaken for 3 hr and then centrifuged at 10000 rpm for 30 min, and supernatants were filtered. All extracts were diluted 10 times with appropriate diluents for analysis of trypsin inhibitor activity (TIA). The method of Roy and Rao (1971) was employed for determining TIA in each extract using Bovine pancreas, type III (Sigma Chemical Co., St. Louis, Mo). Trypsin inhibitor activity was calculated as the number of trypsin units inhibited per mg dry sample.

Thermal stability of trypsin inhibitor activity

Trypsin inhibitor extracts were heated at 95°C for specified times up to 5 hr. Aliquots were removed, cooled rapidly in an ice bath, then subjected to inhibitory activity assay according to the procedure of Roy and Rao (1971).

α-amylase inhibitor activity assay

Amylase inhibitors were extracted according to the method of Cinco et al. (1985). Five g powdered samples were extracted by stirring for 2 hr in 125 mL 0.02M sodium phosphate buffer (pH 6.9) containing 0.15 M Nacl. The extract was centrifuged at 10000 rpm for 15 min. Insoluble matter was discarded and inhibitor activity was measured in the supernatant as described by Deshpande et al. (1982). Porcine pancreatic α-amylase (type 1-A, 2× crystallized Sigma Chemical Co., St. Louis, MO) and bacterial source α-amylase, (BDH) were the sources of enzyme incubated with inhibitor extracts. One unit of enzyme activity was defined as that which liberated from soluble starch 1 mg maltose/min at 37°C and pH 7.0 under the specified conditons.

Thermal stability of α-amylase inhibitor activity

α-amylase inhibitor extracts were heated at 70°C in a water bath for specified times up to 120 min. Aliquots were removed, cooled rapidly in an ice bath, then subjected to inhibitory activity assay according to the procedure of Deshpande et al. (1982).

Phytic acid

Phytic acid content was determined using chromophore reagent as described by Mohamed et al. (1986).

Tannin content

The method of Burns (1971) as modified by Maxson and Rooney (1972) was used: 1-g sample was extracted with 10 mL 1% HCl in methanol for 24 hr at room temperature (≈25°C), with mechanical shaking. After centrifugation at 10000 rpm for 5 min, 1 mL supernatant was

Table 1—Trypsin and α-amylase inhibitors in *Moringa peregrina* (M) and soybean (S) products

Product		Trypsin inhibitor (unit/mg)	α-Amylase inhibitor (unit/g) Pancreatic source	α-Amylase inhibitor (unit/g) Bacterial source
Defatted flour	M	13.0 ± 0.70[b]	246 ± 8.48[b]	235 ± 7.07[b]
	S	26.0 ± 0.01[a]	885 ± 8.49[a]	536 ± 6.36[a]
Protein concentrate	M	14.0 ± 0.71[b]	382 ± 5.66[a]	197 ± 4.24[b]
	S	39.0 ± 2.12[a]	157.0 ± 7.07[b]	368 ± 4.04[a]
Protein isolate	M	5.0 ± 0.70[b]	283 ± 2.24[a]	196 ± 8.49[a]
	S	10.0 ± 1.41[a]	279.4 ± 5.66[a]	217 ± 2.12[a]

[a,b] Means of two determinations on one fraction for M and S for each product in the same column not followed by the same letter are significantly different from each other ($P<0.05$) by Duncan's multiple range test.

mixed with 5 mL vanillin-HCl reagent (equal volumes of 8% concentrated HCl in methanol and 4% vanillin in methanol), and the absorbance at 500 nm was determined after 20 min (LKB Biochrom Ultrospec II, 4040 UV/visible). Different D-catechin concentrations were used as standards. Tannin content was reported as mg D-catechin/g sample.

Chlorogenic acid content

Chlorogenic acid was determined according to the method described by Dorrell (1976). Chlorogenic acid was extracted by refluxing 100 mg sample with 25 mL aqueous 80% ethanol, pH 4.0, for 30 min. Samples were cooled, centrifuged at 2000 rpm for 5 min and volume readjusted to 25 mL. A 0.5 mL aliquot was diluted to 3.0 mL with acidic 80% ethanol. Absorbance was recorded at 330 nm (LKB, Biochrom Ultrospec II, 4040 UV/visible). Different chlorogenic acid concentrations were used as a standard. Results were reported as mg chlorogenic acid/g sample.

In vitro protein digestibility

Fifty mL of an aqueous suspension of sample (6.25 mg protein/mL) in distilled water were adjusted to pH 8.0 with 0.1N HCl and/or 0.1N NaOH. The slurry was then incubated for 15 min at 37°C in a water bath. The multienzyme solution, consisting of 1.6 mg trypsin, 3.1 mg α-chymotrypsin, and 1.3 mg peptidase/mL (enzymes from Sigma Chemical Co., St. Louis, MO), was maintained in an ice bath and adjusted to pH 8.0 with 0.1N HCl and/or 0.1N NaOH. Five mL of multienzyme solution were added to the sample suspension with constant shaking at 37°C. The pH of the suspension after incubation for 10 min at 37°C was recorded and the in vitro digestibility was calculated according to the regression equation of Hsu et al. (1977):

$$Y = 210.464 - 18.103X$$

where Y = *In vitro* digestibility (%); X = pH of the sample suspension after 10 min digestion with multienzyme solution.

Statistical analysis

Data from two replications were evaluated using analysis of variance (Steel and Torrie, 1980) and SAS programs (Statistical Analysis Systems Institute, 1982).

RESULTS & DISCUSSION

Trypsin inhibitor activity

The distribution of trypsin inhibitor contents among fractions of *M.peregrina* and soybean was compared, (Table 1). Trypsin inhibitor activity (TIA) decreased in the following order: protein concentrate > defatted flour > protein isolate. The TIA in *M.peregrina* was lower ($P < 0.05$) than the soybean cultivar tested. Although the presence of TIA in soybeans is well-known, the TIA in *M.peregrina* is presented here for the first time.

Silva et al. (1979) showed that the levels of trypsin inhibitor in 48 cultivars of soybean were 15.3-107 inhibitor units. Other researchers Kakade et al., 1972; Roy and Bhat, 1974) reported different values. The trypsin inhibitor content can be affected by environmental conditions (Ghali, 1988). Trypsin inhibitors can cause pancreatic hypertrophy and are responsible for 30–60% of the growth inhibition in soybean (Tan and Wong, 1982).

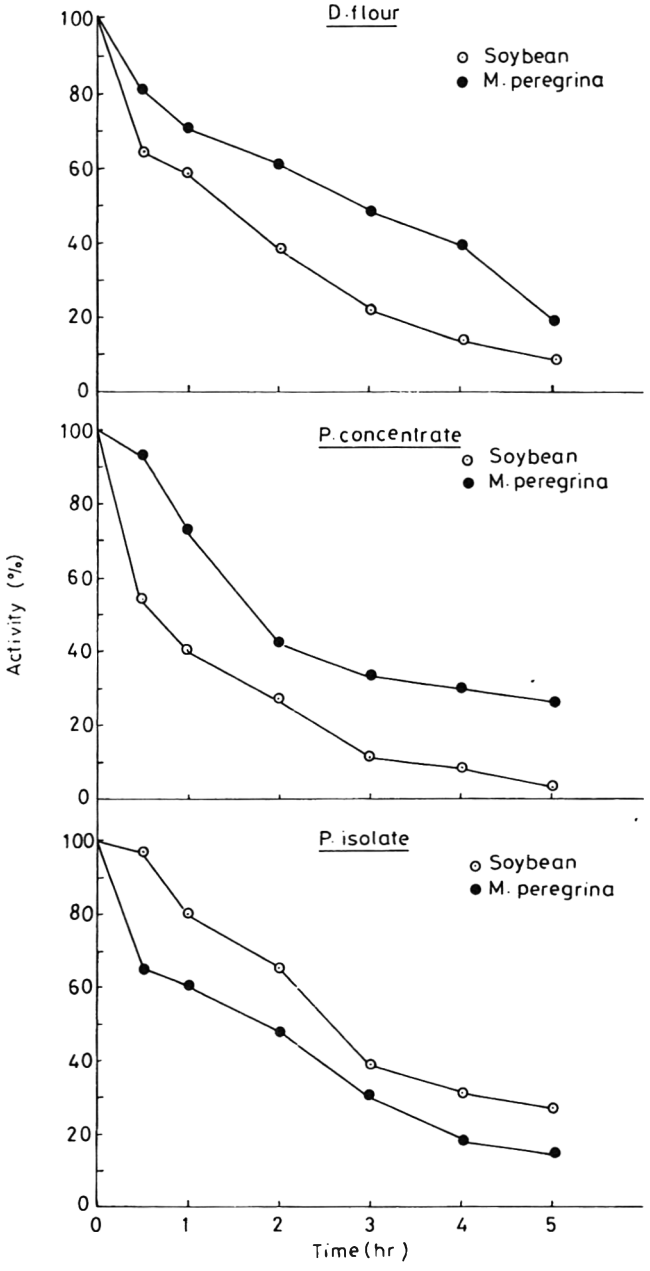

Fig. 1—Effect of heat treatment at 95°C on trypsin inhibitor activity from *M.peregrina* and soybean products.

Therefore, the level of trypsin inhibitor is of importance in the nutritive quality of proteinaceous food components.

Thermal stability of trypsin inhibitor activity

The extracts of defatted flour, protein concentrate, and protein isolate of *M.peregrina*, when boiled for 5 hr at 95°C, retained 18.5%, 26.6%, and 15.8% of the original TIA, respectively. Soybean retained 9.3%, 3.7% and 26.7% in the same order (Fig. 1). Although trypsin inhibitor contents in *M.peregrina* extracts (Table 1) were significantly lower its resistance to heat treatment was higher than that of soybean. Heat processing can cause progressive decrease in TIA but does not achieve zero activity. The residual TIA is either heat-stable activity or an inhibitor that did not interact with the added trypsin under the specified conditions (Smith et al., 1980). Koeppe et al. (1985) found that 20% of the original amaranth TIA remained at the end of 7 hr heating at 100°C.

α-Amylase inhibitory activity

The α-amylase inhibitor activity (AIA) of *M.peregrina* and soybean was investigated using pancreatic and bacterial amylase (Table 1). AIA in *M.peregrina* defatted flour was lower ($P < 0.05$) than in soybean defatted flour and AIA was generally greater for pancreatic amylase than for bacterial amylase. In contrast to trypsin inhibitors, α-amylase inhibitors are not as well understood (Granum, 1979). Several researchers (Powers and Whitaker, 1977; Frels and Rupnow, 1984; Cinco et al., 1985) have studied α-amylase inhibitor activity in legumes, cereals and other plants. According to Törrönen et al. (1992), the rye α-amylase inhibitor could be considered a potential α-amylase inhibitor in breadmaking when flour made of sprout-damaged rye is used. The extent of inhibition of different amylases depends on pH during the interaction of enzymes and inhibitor and on the time of interaction between inhibitor and enzyme (Sharma and Pattabriman, 1982).

Thermal stability of α-amylase inhibitor

Gradual loss in inhibitory activity of pancreatic and bacterial α-amylases was affected by heat treatment (Fig. 2) for 120 min at 70°C. For *M.peregrina*, the loss (as measured with pancreatic α-amylase) was more than 75% (in protein concentrate and protein isolate) and 50% (in defatted flour) of original activity. More than 60%, 65%, and 50% of the inhibitory activity (as measured with bacterial α-amylase) in defatted flour, protein concentrate, and protein isolate, respectively, were lost in 120 min at 70°C. Generally, the bacterial α-amylase inhibitor of *M.peregrina*, was more heat resistant than that of soybean, but the pancreatic α-amylase inhibitor in M.peregrina was less heat resistant than soybean.

Sharma and Pattabriaman (1982) found that α-amylase inhibitor from Yam was fairly heat stable and was active against human salivary, human pancreatic and pig pancreatic amylase.

Phytic acid

Phytic acid contents in *M.peregrina* and soybean products were compared (Table 2). The highest phytic acid content was found in *M.peregrina* protein concentrate. This could be a result of its higher ($P < 0.05$) concentration in defatted flour, or may be due to a concentration effect during processing.

Phytic acid can reduce availability of divalent cations (Tan et al., 1983; Khetarpaul and Chauhan, 1991). Phytic acid is among the compounds that have been proposed as toxic constituents (Storey et al., 1983). Thompson and Erdman (1982) reported 1.54%, 0.88%, 1.08%, 1.96%, 1.63%, and 1.82% values for dehulled soybean, hypocotyles, tempeh, tofu, soy isolate, and defatted flour, respectively. A value of 1.52% was reported by deBoland et al. (1975) for soy isolate. Concentrations of phytate in soybean from our results (1.3 to 2.1%) were similar to those of other investigations.

Tannin content

Tannin contents of *M.peregrina* and soybean fractions were also compared (Table 2). Levels were generally very low and not of nutritional concern. Tannins (phenolic compounds) are nonspecific inhibitors of enzymes and may reduce protein quality by directly complexing with food proteins (National Academy of Sciences, 1973). High levels of tannins have been recognized to have nutritionally harmful effects (Jambunathan and Mertz, 1973).

Chlorogenic acid

The contents of chlorogenic acid (CA) in *M.peregrina* and soybean fractions were also compared (Table 2). Low levels of CA occurred particularly in M.peregrina fractions. Chlorogenic acid is the major phenolic compound in sunflower (Pomenta and Burns, 1971; Sabir et al., 1974) and it functions as part of the oxidase system in the plant (Hanson and Zucker, 1963). Dorrell (1976) reported from 1.1 to 4.5% CA in sunflower seeds. The presence of CA in plant protein concentrates affects protein digestibility by inhibiting the reactivity with proteolytic enzymes (Lahiry and Satterlee, 1975; Lahiry et al., 1977). It also affects final acceptability, and discoloration of the meal in an alkaline environment (Carter et al., 1972; Sosulski, 1979).

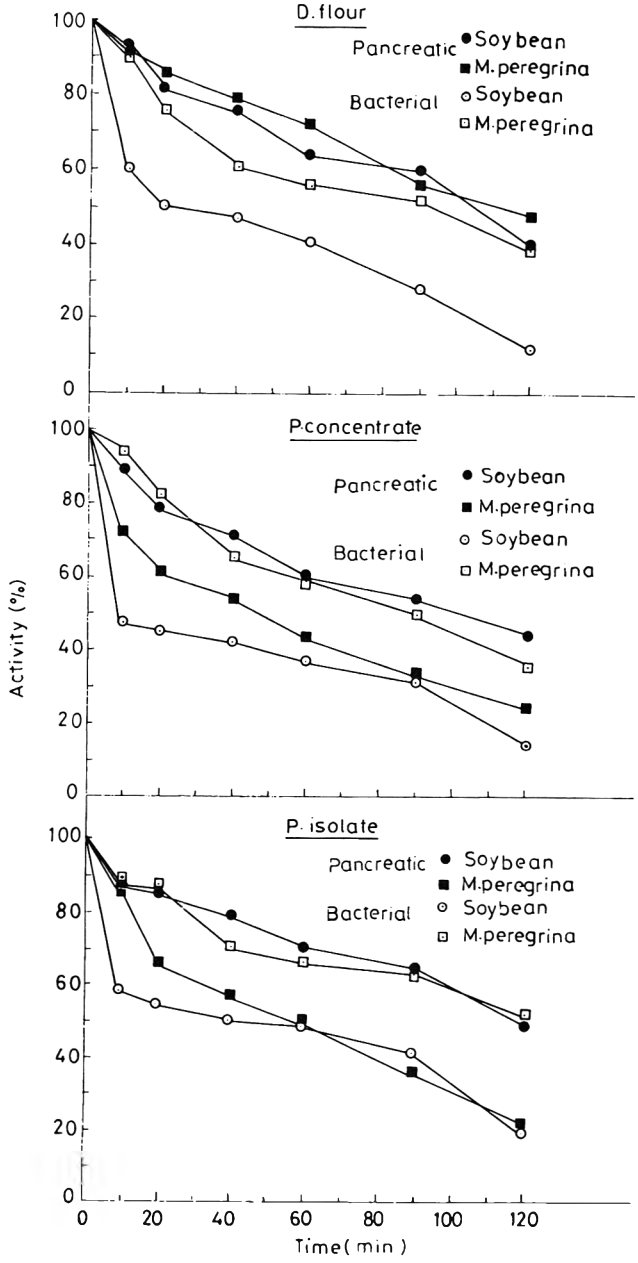

Fig. 2—Effect of heat treatment at 70°C on α-amylase inhibitor from *M.peregrina* and soybean product.

In vitro digestability

The *in vitro* protein digestibility (IVPD) of *M.peregrina* and soybean products were also compared (Table 2). Generally, the IVPD indicated that a high percentage of protein was readily digested. However, *M.peregrina* products were less digestible ($P < 0.05$) than soybean. The IVPD for soybean products was similar to previously reported values for such products (Hsu et al., 1977; Bryant et al., 1988).

Table 2—Phytic ac d, tannin and chlorogenic acid content in *Moringa peregrina* (M) and soybean (S) products and their In Vitro digestibility*

Product		Phytic acid (mg/g)	Tannin (mg catechin/g)	Chlorogenic acid (mg/g)	Digestibility (%)
Defatted flour	M	26.00 ± 0.61[a]	0.15 ± 0.01[b]	0.97 ± 0.01[b]	69.72 ± 0.64[a]
	S	21.00 ± 0.05[b]	0.32 ± 0.01[a]	2.50 ± 0.09[a]	76.32 ± 2.30[a]
Protein concentrate	M	42.16 ± 0.55[a]	0.05 ± 0.01[b]	0.85 ± 0.01[b]	80.13 ± 0.52[b]
	S	14.67 ± 0.33[b]	0.09 ± 0.01[a]	1.67 ± 0.03[a]	87.27 ± 0.13[a]
Protein isolate	M	9.87 ± 1.20[a]	0.13 ± 0.01[a]	1.07 ± 0.04[b]	82.48 ± 0.52[b]
	S	13.28 ± 0.21[a]	0.15 ± 0.01[a]	1.93 ± 0.04[a]	88.45 ± 0.26[a]

[a,b] Means of two determinations on one fraction for M and S for each product in the same column not followed by the same letter are significantly different from each other ($P<0.05$) by Duncan's multiple range test.

CONCLUSION

THE RESULTS suggest the suitability of *M.peregrina* products as new viable protein sources. The relatively lower digestibility or nutritional properties of *M.peregrina* proteins might be improved by other processes such as application of heat (autoclaving, roasting, soaking, steaming), enzyme modification, or ion exchange treatment.

REFERENCES

Al-Kahtani, H.A. 1993. *Moringa peregrina* (Al-Yassar or Al-Ban) seeds oil from Northwest Saudi Arabia. J. King Saud Univ. (Agricultural Sciences). In press.

Al-Kahtani, H.A. and Abou-Arab, A.A. 1993. Comparison of physical, chemical, and functional properties of Moringa peregrina (Al-Yassar or Al-Ban) and soybean proteins. Cereal Chem. 70: 619–626.

Al-Yahya, M.A., Al-Meshal, I.A., Mossa, J.S., Al-Bader, A.A. and Tariq, M. 1990. *Saudi Plants: A Phytochemical and Biological Approach*. p. 288–290. King Abdulaziz City for Science & Technology, Riyadh, Saudi Arabia.

Bryant, L.A., Montecalvo, J. Jr., Morey, K.S., and Loy, B. 1988. Processing, fuctional, and nutritional properties of okra seed products. J. Food Sci. 53: 810–816.

Burns, R.E. 1971. Methoc for estimation of tannin in grain sorghum. Agronomy J. 63: 511–512.

Carter, C.M., Gheyasuddin, S., and Mattil, K.F. 1972. The effect of chlorogenic, quinic, and coffe:c acids on the solubility and color of protein isolates, especially from sunflower seed. Cereal Chem. 49: 508–514.

Cinco, F., Frels, J.M., Holt, D.L., and Rupnow, J.H. 1985. Determination of the number and heat stability of α-amylase inhibitors in white and red kidney bean (phaseolus vulgaris). J. Food Sci. 50: 533–534.

de Boland, A.R., Garner, G.B., and O'Dell, B.L. 1975. Identification and properties of phytate in cereal grains and oilseed products. J. Agri. Food Chem. 23: 1188.

Deshpande, S.S., Sathe, S.K., Salunkhe, D.K., and Cornforth, D.P. 1982. Effects of dehulling on phytic acid, polyphenols, and enzyme inhibitors of dry beans (phaseolus vulgaris L.). J. Food Sci. 47: 1846.

Dorrell, D.G. 1976. Chlorogenic acid content of meal from cultivated and wild sunflowers. Crop Sci. 16: 422–424.

Erdman, J.W.Jr., Weingartner, K.E., Mustakas, G.C., Schmutz, R.D., Parker, H.M., and Forbes, R.M. 1980. Zinc and magnesium bioavailability from acid-precipitated and neutralized soybean protein products. J. Food Sci. 45: 1193.

FAO. 1988. Traditional Food Plants. Food and Agriculture Organization, p. 369–373. FAO Food and Nutrition paper 42, Rome, Italy.

Frels, J.M. and Rupnow, J.H. 1984. Purification and partial characerization of two α-amylase inhibitors from black bean (*Phaseolus vulgaris*). J. Food Bio. 8: 281.

Ghali, A.B.S. 1988. The effect of environmental conditions on the chemical composition of soybean seeds: Relationship between the protein, oil, carbohydrate and trypsin inhibitor content. Food Chem. 29: 221–231.

Granum, P.E. 1979. Studies on α-amylase inhibitors in foods. Food Chem. 4: 173.

Hanson, K.R. and Zucker, M. 1963. The biosynthesis of chlorogenic acid related conjugates of the hydroxycinnamic acid. J. Bio. 238: 1105.

Hsu, H.W., Vavak, D.L., Satterlee, L.D., and Miller, G.A. 1977. A mutlenzyme technique for estimating protein digestibility. J. Food Sci. 42: 1269–1273.

Jambunathan, R. and Mertz, E.T. 1973. In: price, M.L. and Butter, L.G. 1977. Rapid visual estimation and spectrophotometric determination of tannin content of sorghum grain. J. Agri. Food Chem. 25: 1268–1273.

Kakade, M.L., Hoffa, D.E., and Liener, I.E. 1973. Contribution of trypsin inhibitors to the deleterious effects of unheated soybeans fed to rats. J. Nutr. 103: 1772.

Khetarpaul, N. and Chauhan, B.M. 1991. Effect of natural fermentation on antinutrients and in vitro digestability of starch and protein in pearl millet flour. J. Sci. Food Agri. 55: 189–195.

Koeppe, S., Rupnow, J.H., Walker, C.E., and Davis, A. 1985. Isolation and heat stability of trypsin inhibitors in amaranth (*Amaranthus hypochondriacus*). J. Food Sci. 50: 1519–1521.

Kumar, A. and Chauhan, B.M. 1993. Effect of phytic acid on protein digestability (*In vitro*) and Hcl-extractability of minerals in peorl millet sprouts. Cereal Chem. 70: 504–506.

Lahiry, N.L. and Satterlee, L.D. 1975. Release and estimation of chlorogenic acid in leaf protein concentrate. J. Food Sci. 40: 1326.

Lahiry, N.L., Satterlee, L.D., Hsu, H.W., and Wallace, G.W. 1977. Characterization of the chlorogenic acid binding fraction in leaf protein concentration. J. Food Sci. 42: 83–85.

Mameesh, M.S. and Tomar, M. 1993. Phytate content of some popular Kuwaiti foods. Cereal Chem. 70: 502–503.

Mattil, K.F. 1974. Compositonal, nutritional and functional properties, and quality criteria of soy protein concentrates and soy protein isolates. J. Amer. Oil Chem. Soc. 15: 81A–84A.

Maxon, E.C. and Rooney, L.W. 1972. Two methods of tannin analysis for sorghum bicolor (L.) moench grain. Crop Sci. 12: 253–254.

Migahid, A.M. 1978. *Flora of Saudi Arabia*, 2nd ed., p. 101. King Saud Univ., Riyadh, Saudi Arabia.

Mohamed, A., Perera, P.J., and Hafez, Y.S. 1986. New chromophore for phytic acid determination. Cereal Chem. 63: 475–478.

National Academy of Sciences. 1973. *Toxicants Occurring Nationally in Foods*. National Academy of Sciences, Washington, DC.

Ologhobo, A.D. and Fetuga, B.L. 1984. Distribution of phosphorus and phytate in some Nigerian varieties of legumes and some effects of processing. J. Food Sci. 49: 199–201.

Pomenta, J.V. and Burns, E.E. 1971. Factors effecting chlorogenic, quinic and caffeic acid levels in sunflower kernels. J. Food Sci. 36: 940.

Powers, J.R. and Whitaker, J.R. 1977. Purification and some physical and chemical properties of red kidney bean (*phaseolus vulgaris*) α-amylase inhibitor. J. Food Biochem. 1: 217.

Roy, D.N. and Rao, P.S. 1971. Evidence, isolation, purification and some properties of a trypsin inhibitor in lathyrus sativus. J. Agri. Food Chem. 19: 257.

Roy, D.N. and Bhat, R.V. 1974. Trypsin inhibitor content in some varieties of soya bean (*Glycine max* L.) and sunflower seeds (*Helianthus annuus* L.). J. Sci. Food Agri. 25: 765–769.

Sabir, M.A., Sosulski, F.W., and Kernan, J.A. 1974. Phenolic constituents in sunflower flour. J. Agri. Food Chem. 22: 572–574.

Sharma, K.K. and Pattabiraman, T.N. 1982. Natural plant enzyme inhibitors. Purification and properties of an amylase inhibitor from yam (*Dioscorea alata*). J. Sci. Food Agri. 33: 255–262.

Silva, A.D., Barbosa, C.F., and Portela, F. 1979. In: Ghali, A.B.S. 1988. The effect of environmental conditions on the chemical composition of soybean seeds: Relationship between the protein, oil, carohydrate and trypsin inhibitor content. Food Chem. 29: 221–231.

Smith, C., Megen, W.V., Twaalfhoven, L., and Hitchcock, C. 1980. The determination of trypsin inhibitor levels in foodstuffs. J. Sci. Food Agri. 31: 341–350.

Somali, M.A., Bajneid, M.A., and Fhaimani, S.S. 1984. Chemical composition and characteristics of Moringa peregrina seeds and seeds oil. J. Amer. Oil Chem. Soc. 61: 85–86.

Sosulski, F.W. 1979. Organoleptic and nutritional affects of phenolic compounds on oliseed protein products: A review. J. Amer. Oil Chem. Soc. 56: 711.

Sosulski, F.W., Chakraborty, P., and Humbert, E.S. 1978. Legume-based imitation and blended milk products. Can. Ins. Food Sci. Technol. J. 11: 117.

Statistical Analysis Systems Institute. 1982. *Statistical Analysis System Users Guide*. SAS Institute, Inc., Cary, NC.

Steel, R.G.D. and Torrie, J.H. 1980. *Principles and Procedures of Statistics*. McGraw Hill Book Company, Inc., New York.

Storey, R., Bower, N., Lovejoy, C., and Tagget, R. 1983. Analysis of selected nutritional and antinutritional factors in Jojoba seed from the United States and Mexico. In *Jojoba and Its Uses*. Proc. Int. Conf. 5th, A.E. Cesnick (Ed.), p. 25. Univ. of Arizona, Tucson, AZ.

Tan, N.H. and Wong, K.C. 1982. Thermal stability of trypsin inhibitor activity in winged bean (*Psophocarpus tetragonolobus*). J. Agri. Food Chem. 30: 1140–1143.

Tan, N.H., Rohim, Z.H.A., Khor, H.T., and Wong, K.C. 1983. Winged bean (Psophocarpus tetragonolobus) tannin level, phytate content, and hemagglutinating activity. J. Agri. Food Chem. 31: 916–917.

Thompson, D.B. and Erdman, J.W.Jr. 1982. Phytic acid determination in soybeans. J. Food Sci. 47: 513–517.

Törrönen, A., Leisola, M., and Haarasilta, S. 1992. Inhibition of rye α-amylase activity by barley-amylase inhibitor. Cereal Chem. 69: 355–358.

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Calcium-Induced Destabilization of Oil-in-Water Emulsions Stabilized by Caseinate or by β-Lactoglobulin

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ABSTRACT

The stability to aggregation of 20% soya oil-in-water emulsions stabilized by 0.3 to 2% sodium caseinate or β-lactoglobulin in the presence of calcium chloride solutions was studied using light scattering and electron microscopy. Stability increased with the amount of protein in the emulsion, and decreased with the concentration of added calcium. Growth of particle size with concentration of Ca^{2+} was more in emulsions containing lower concentrations of protein. Sodium chloride at 50 and 100 mM stabilized both systems to the presence of calcium ions. Microstructure and light scattering showed caseinate emulsions formed clusters even at low concentrations of Ca^{2+} while β-lactoglobulin emulsions formed extensive strands.

Key Words: emulsions, caseinate, β-lactoglobulin, calcium, flocculation

INTRODUCTION

THE PHYSICOCHEMICAL ASPECTS of interactions between calcium and proteins have been extensively studied (Zittle et al., 1957; Baumy and Brulé, 1988; Patocka and Jelen, 1991). Likewise, several studies have been performed on the binding of cations, especially Ca^{2+}, to milk proteins under a variety of environmental conditions (Dalgleish and Parker, 1980; Parker and Dalgleish, 1981; deWit and Klarenbeek, 1984; Pappas and Rothwell, 1991). The knowledge from those studies is very useful in industrial dairy processing operations, notably cheesemaking (Marshall and Green, 1980) and food product development (Mulvihill and Kinsella, 1988).

Milk proteins are well known surfactants in food emulsion systems (Tornberg, 1978a; Mulvihill, 1992). They stabilize oil-in-water emulsions and foams because they are amphiphiles (Parker, 1988). They adsorb to oil-water interfaces and stabilize emulsions by a combination of charge repulsion and steric stabilization (Dickinson and Stainsby, 1982).

The association of Ca^{2+} with adsorbed proteins has been studied less than binding in solution. Most studies have generally been focused on the use of individual purified proteins in model systems, often adsorbed to latex particles or hydrocarbon oil rather than triglycerides (Dalgleish et al., 1985; Dickinson et al., 1987, 1992.). Such model systems may be very different from true food emulsions; therefore, we undertook the study of emulsions containing emulsifiers and triglyceride oil typical of those commonly employed in commercial food preparations. Experimental evidence on the details of destabilization by added salts is not extensive, and we studied the effects of combinations of Ca and NaCl on the stability of these emulsion particles.

Light scattering is a technique for determining particle size distribution of emulsions (Dickinson and Stainsby, 1987). Microscopy has also been utilized for the study of emulsion destabilization (Vincent, 1974; Britten and Giroux, 1991; Dickinson et al., 1992). We used these techniques to provide more insight into the effect of the addition of Ca^{2+} to protein emulsifiers at the soya oil-water interface in emulsions containing 20% (v/v) oil. Particularly, we focused on destabilization and aggregation behavior of emulsion droplets stabilized by caseinate, a protein complex which shows Ca^{2+}-dependent aggregation, compared with β-lactoglobulin (β-lg), which does not. Our objective was to study the effects of changing the concentration of Ca^{2+} and the ionic strength on the stability of emulsions containing different concentrations of the two different proteins.

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MATERIALS & METHODS

Materials

Sodium caseinate was prepared from skim milk by acid precipitation with 1M HCl to pH 4.6. The acid casein was washed with distilled water and then redissolved to pH 7 using 1M NaOH. Finally, the product was freeze-dried. Soya oil, β-lg and other chemicals were purchased from Sigma Chemical Co. (St. Louis, MO) and used without further purification. The protein solutions for emulsion preparation were obtained by dissolving 4g of the protein in 20 mM imidazole/HCl buffer solution, at pH 7, containing 0, 50 or 100 mM NaCl, and making up to 100 mL. These solutions were then filtered through a 0.22-μm filter (Millipore Canada Ltd., Mississauga, Ontario).

Emulsion preparation

Soya oil-in-water emulsions stabilized with proteins were prepared by homogenizing 4 mL of soya oil, and 16 mL of solutions of defined protein concentrations made by diluting the standard protein solution with imidazole/HCl buffer. Homogenization was performed using a model 110S Microfluidizer (Microfluidics Corp., Newton, MA) with an input pressure of 0.2 MPa; this corresponds to a pressure drop of 28 MPa during homogenization. Final protein concentrations in the emulsions were in the range 0.3–2% (w/v) while the soya oil content was constant at 20% (v/v) in all emulsions. Each final emulsion was prepared by passing it 6 times through the Microfluidizer. Three sets of emulsions were prepared in this way containing 0, 50 and 100 mM NaCl in the buffer solutions with a range of protein concentrations.

Addition of calcium to emulsions

The effect of adding Ca^{2+} on the emulsions was studied by pipetting 0.5 mL of the emulsion into a 1.5-mL centrifuge tube placed in a thermostatically controlled water bath at 30°C. To this, sufficient amounts of 50 mM $CaCl_2$ solution in imidazole buffer were added to obtain final concentrations of $CaCl_2$ in the range 2–16 mM for emulsions containing caseinate and up to 25 mM for emulsions containing β-lg. The mixture was shaken for about 1 min, and then the size distribution was determined. Increasing the reaction time from 1 min up to 30 min did not cause any appreciable change in particle size distribution or average sizes of emulsion aggregates, i.e., an equilibrium state of aggregation was established under these experimental conditions.

Particle analysis

The droplet size distribution and average size of particles in stable and destabilized emulsions were measured by light scattering using a Mastersizer X (Malvern Instruments Inc., Southboro, MA). The presentation factor was 0303 (i.e., refractive index and absorption of 1.414 and 0.001, respectively). Concentrated emulsion samples could not be measured, and had to be diluted. For this we chose a buffer which would maintain intact the aggregated particles which were formed from the action of Ca^{2+}. For the duration of the measurement (about 1 min) 20 mM imidazole buffer containing 50 mM NaCl and 5 mM $CaCl_2$ (pH 7) at 30°C was sufficient. This buffer solution was prepared and filtered through a 0.22-μm filter before the emulsion samples were added.

Microstructure studies were carried out on emulsions containing 1% protein, either untreated or in the presence of 10 mM Ca^{2+}, but without

added NaCl. Cryo-scanning electron microscopy (Cryo-SEM) was performed using the Emscope SP 2000A cryo unit (Emscope Laboratories Ltd, Ashford, England). The filter paper method (SP2000 Sputter Cryo Evaluation Reports, 1982) was used. The sample was frozen using liquid nitrogen slush at about −207°C after which it was transferred to the preparation chamber under vacuum for freeze-etching and sputter coating with gold to about 2 nm. Examination of the sample was done on the cold stage (operating at <−130°C) of a Hitachi S-750 SEM (Hitachi Ltd., Tokyo, Japan) operating at an accelerating voltage of 10 kV.

RESULTS

THE WEIGHT AVERAGE PARTICLE SIZES (d_{43}) in emulsions produced by the Microfluidizer, and stabilized by β-lg (Fig. 1a) and by caseinate (Fig. 1b) were compared at different concentrations of protein and NaCl in the absence of Ca^{2+}. Results for emulsions containing caseinate show that the particle size decreased as concentration of protein increased, in agreement with earlier results using similar preparations in the absence of NaCl (Fang and Dalgleish, 1993). Although differences in particle size were significant (5% level) for protein concentrations of 0.5% and below, no significant differences occurred between results at higher concentrations of protein, where particle sizes were smaller. The presence of NaCl at 50 or 100 mM made no significant alteration in sizes of particles in emulsions containing caseinate. Conversely, emulsions prepared using β-lg showed a generally trend of increasing particle size with concentration of added salt, at all concentrations of protein, suggesting that the emulsifying capacity of β-lg was decreased as ionic strength increased. This salt effect was significant ($P \leq 5\%$) for all protein concentrations <1%, and we therefore assumed that similar trends at higher concentrations of protein were also significant.

Effects of addition of Ca^{2+} on the average particle sizes in oil-in-water emulsions prepared using different concentrations of β-lg (Fig. 2) and caseinate (Fig. 3), were also compared. In general, increasing protein content made the emulsions more stable after addition of Ca^{2+}. Not only was the critical concentration of Ca^{2+} required for destabilization increased by the presence of greater amounts of protein, but the rate of change of particle size with concentration of Ca^{2+} was greater at lower protein concentrations. Destabilization of these emulsions by Ca was not simply the effect of ionic strength, since increasing ionic strength by adding NaCl, at a fixed concentration of Ca^{2+}, inhibited the Ca^{2+}-induced aggregation. For the emulsion containing 0.5% β-lg, if the critical concentration of $CaCl_2$ was 5 mM in the absence of NaCl (ionic strength 15 mM), we would expect that addition of 50 mM NaCl would also cause destabilization (combined ionic strength 65 mM). The observed effect was the opposite.

In the presence of even 50 mM NaCl, emulsions stabilized by β-lg remained almost completely stable in the presence of concentrations of Ca^{2+} as high as 25 mM. Conversely, in the absence of added NaCl, the emulsions containing 1% protein were destabilized by low concentrations of Ca^{2+} (about 5 mM). At high protein concentrations (1.5 and 2%), addition of Ca^{2+} had a much smaller effect (Fig. 2). No significant differences occurred between 50 and 100 mM added NaCl on the behavior of the emulsions. Destabilization did not increase linearly with concentration of added Ca^{2+}. The particle size reached a plateau at concentrations of Ca^{2+} which depended on the concentration of protein, above which no further increase in particle size was observed with added Ca^{2+}.

Emulsions containing casein behaved rather differently. NaCl had a progressive stabilizing effect, which increased with concentration (Fig. 3) rather than the complete stabilizing effect of NaCl, which was found in emulsions containing β-lg where the presence of any salt was adequate to confer stability. A second difference was that particle sizes in the emulsions prepared with caseinate continued to increase as concentrations of Ca increased, contrasted to the limiting plateau values found for β-lg. However, for emulsions containing no added NaCl, the

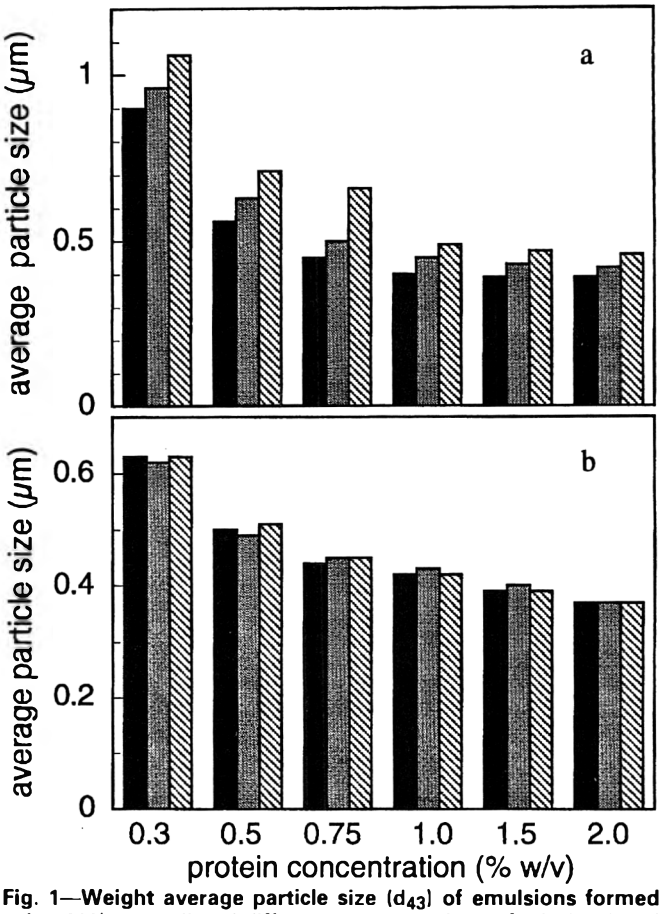

Fig. 1—Weight average particle size (d_{43}) of emulsions formed using 20% soya oil and different concentrations of β-lg (top) and caseinate. Each set of three bars represents experiments at concentrations of added NaCl of 0 mM (filled bar), 50 mM (dotted bar) and 100 mM (hatched bar), respectively. Each result is the average of measurements on three separate emulsions.

amount of Ca^{2+} required to initiate instability (2–3 mM in solutions containing 0.3 and 0.5% protein) was the same whether they contained β-lg or caseinate. As found for emulsions containing β-lg, increasing concentration of casein enhanced stability, since an increasing concentration of Ca^{2+} was required to initiate precipitation. Unlike β-lg, emulsions containing caseinate were not very stable at any concentrations of NaCl, once the critical concentration of Ca^{2+} for the protein content and ionic strength had been exceeded. The sizes of particles in the destabilized caseinate emulsions were considerably larger than those in destabilized β-lg emulsions. As with β-lg, the effect of Ca^{2+} was not simply to provide a high ionic strength, since the stability of the emulsion increased when NaCl was present. Emulsions, when suspended in solutions of the same ionic strength, but containing different proportions of Ca^{2+} and Na^{+}, behaved differently. The effect of NaCl seemed rather to reduce the binding of Ca^{2+} to casein, or to β-lactoglobulin, in the emulsion.

The typical particle size distributions (Fig. 4) measured in emulsions containing 1% caseinate or β-lg at various concentrations of Ca^{2+}, emphasize the different mechanisms of destabilization. Once destabilized, emulsions containing caseinate showed a general reduction in proportion of emulsion droplets in the original size range and an increase in proportion of large particles. This gave a bimodal size distribution as concentration of $CaCl_2$ increased. However, the shape of the size distribution of small particles hardly changed although some very large particles were formed from aggregation of small ones. Very few particles appeared in the intermediate size range (1–4 μm) between the aggregate and original particles. However, emulsions with β-lg showed monomodal size distributions which only shifted to greater average size as concentration of $CaCl_2$ in-

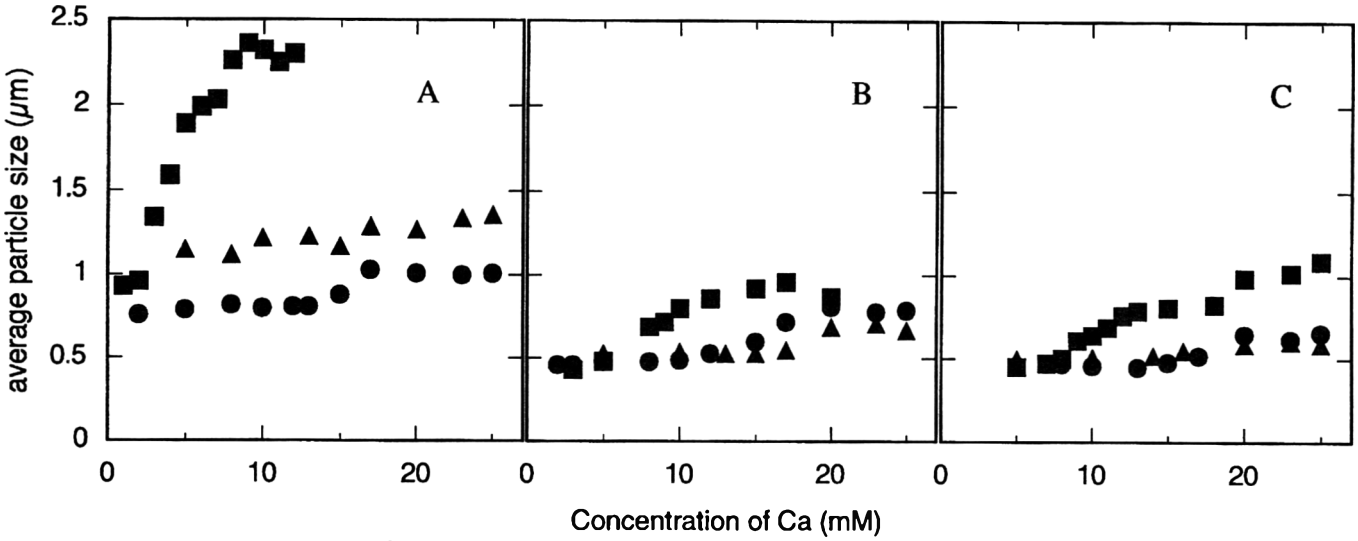

Fig. 2—Effects of concentration of Ca^{2+} on the particle sizes of droplets in emulsions (20% soya oil) prepared using β-lg. Emulsions prepared at three different protein concentrations are shown: (A) 0.3%; (B) 1% and (C) 2% protein, and for each protein concentration the effects of three concentrations of added NaCl are shown by ■, 0 mM; ●, 50 mM; ▲, 100 mM. Each result is the average of measurements on three separate emulsions.

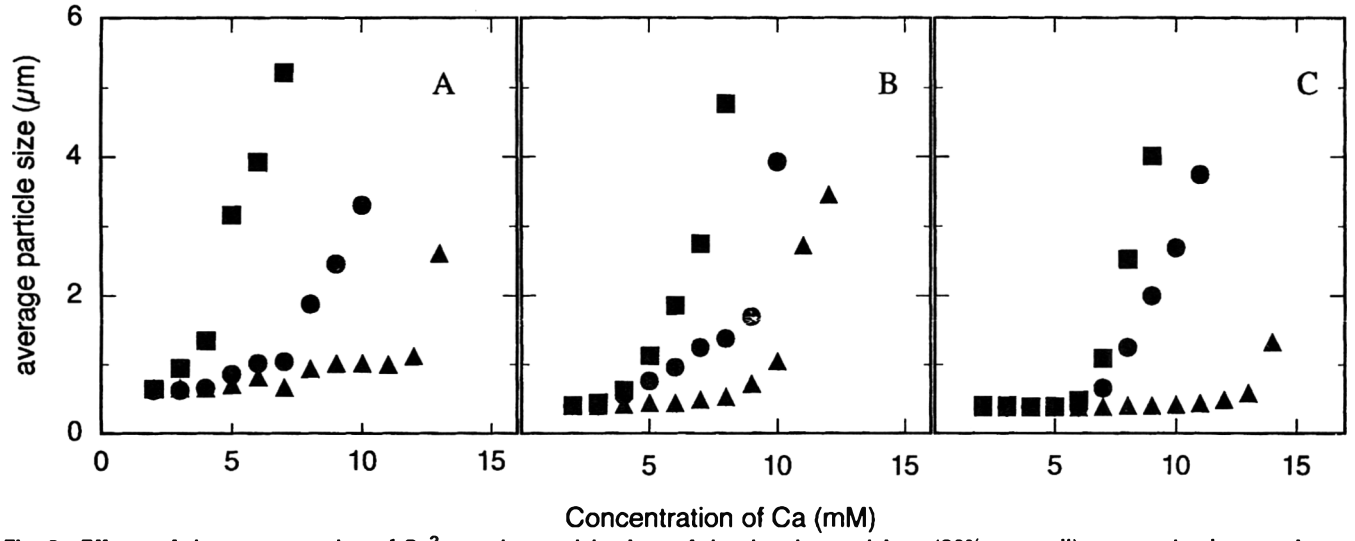

Fig. 3—Effects of the concentration of Ca^{2+} on the particle sizes of droplets in emulsions (20% soya oil) prepared using caseinate. Emulsions prepared at three different protein concentrations are shown: (A) 0.3%; (B) 1% and (C) 2% protein, and for each protein concentration the effects of three concentrations of added NaCl are shown by ■, 0 mM; ●, 50 mM; ▲, 100 mM. Each result is the average of measurements on three separate emulsions.

creased. No large particles (above 5μm) occurred in these emulsions even after destabilization.

A critical concentration of Ca^{2+} is readily defined (Fig. 3) which is required for destabilization of the emulsions, at any concentration of casein and ionic strength. These critical concentrations of Ca^{2+} (Fig. 5) were measured by determining the concentration of Ca^{2+} at which visible flocculation of the emulsion occurred. This illustrates the increased stability to Ca^{2+}-destabilization which was observed with increasing added NaCl, as well as the generally stabilizing effect of increasing protein. There was, however, a reproducible indication that emulsions formed with 0.3% protein were somewhat more stable than those formed with 0.5% protein, especially in the presence of NaCl.

To provide a visual perspective of the aggregation, and to investigate whether emulsion droplets were coalescing or merely flocculating, we made micrographic studies of both stable emulsions and Ca^{2+}-destabilized emulsions (Fig. 6). The stable emulsions were both in single droplets as well as in various states of limited flocculation, presumably induced by sample preparation. The mechanism of aggregation of the protein-stabilized emulsions in the presence of Ca^{2+} was flocculation, rather than coalescence, judging from the globular shapes of individual particles within the aggregate (partial coalescence did not occur since the oil in the emulsions was liquid). The degrees of association and the structures of destabilized emulsions containing caseinate, however, appeared to be different from those obtained for β-lg emulsions and this agreed with the trend from particle size distributions (Fig. 4). Emulsions with caseinate formed amorphous aggregated particles, whereas those containing β-lg appeared to produce fine strands of aggregate. The reason, therefore, that only small aggregates were seen in the light-scattering equipment was that the samples were circulated through the sample cell, and the shearing action broke the fine-stranded aggregates but apparently not the more compact flocs formed from caseinate-stabilized emulsions.

DISCUSSION

TRENDS (Fig. 1) show that β-lg was slightly less effective than caseinate in stabilizing emulsion droplets as they were being formed, especially in solutions of increased ionic strength. Torn-

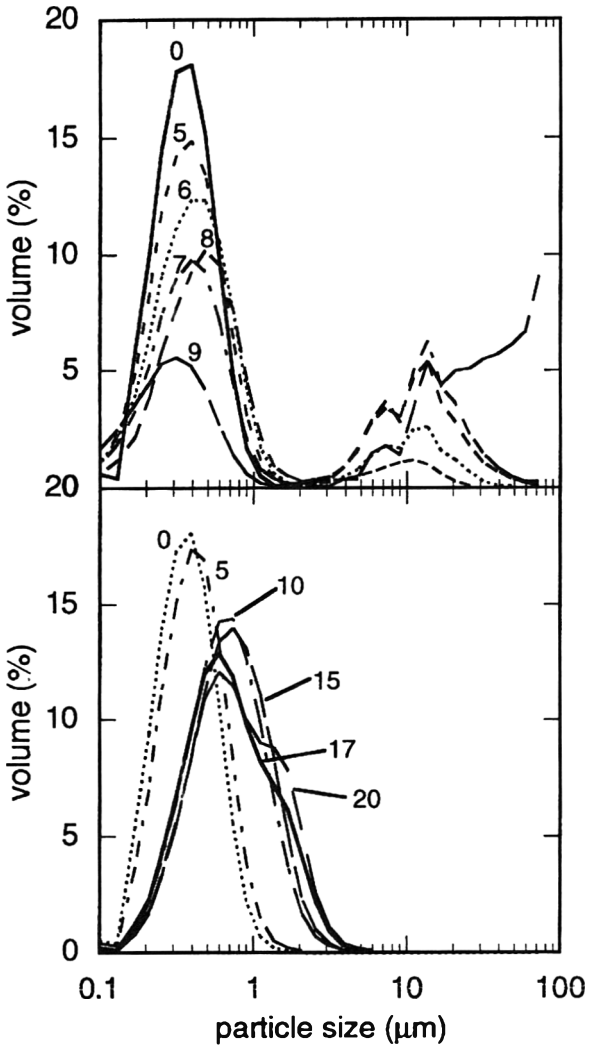

Fig. 4—Typical size distributions of the particles in emulsions containing 20% soya oil and 1% caseinate (top) or β-lg (bottom), in buffer containing 50 mM NaCl, at the different concentrations of $CaCl_2$ marked on the graphs.

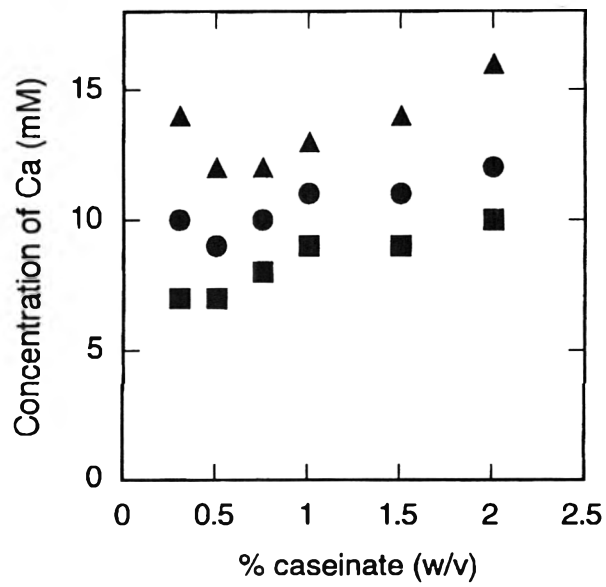

Fig. 5—Concentrations of $CaCl_2$ at which the reaction with caseinate emulsions became too rapid to be measured using the Mastersizer. This coincided with visible precipitates being formed at these concentrations. The concentrations of added NaCl in the experiments were: ■, 0 mM; ●, 50 mM; ▲, 100 mM.

berg (1978b) reported that 0.2M NaCl reduced adsorption of whey protein concentrate on a soya oil/water interface in emulsions formed using a valve homogenizer. Probably NaCl had a negative effect on the adsorption of β-lg (the major whey protein) to the oil-water interface.

Higher average particle sizes at low concentrations of proteins occurred partly because insufficient surfactant causes droplets to coalesce to reduce interfacial area and maximize surface coverage. The flocculation seen by electron microscopy in untreated emulsions (Fig. 6) may have been partly bridging flocculation which occurred during homogenization. Vincent (1974) reported that emulsions formed using high-molecular-weight surfactants like proteins had more tendencies to associate by bridging compared to low-molecular-weight surfactants. This may, contribute to the polydispersity of emulsion droplets. This observation may help explain the size of aggregates formed when Ca^{2+} ions were added to the emulsions, especially with no NaCl. For both protein-stabilized emulsions, a lower concentration of Ca^{2+} was required to aggregate emulsions formed with 0.3 and 0.5% protein and, also, the rate of change of particle size was higher with increasing Ca^{2+}.

Binding of Ca^{2+} to caseins has been attributed to the presence of phosphoserine groups in individual casein molecules (Parker and Dalgleish, 1981; Horne and Dalgleish, 1980; Swaisgood, 1992). The general effect of ionic strength, resulting in destabilization of the DLVO mechanism for particle stability, may have some effect on the emulsion stability. However it was not adequate to explain the destabilization by Ca^{2+} of casein micelles in solution (Payens, 1979) and caseinate-stabilized emulsions (Dickinson et al., 1987). It may be important in the aggregation of individual caseins (Horne and Dalgleish, 1980). The specific binding of Ca^{2+} to caseinate is most likely responsible for the decrease in emulsion stability with increasing $CaCl_2$. The increased stability of the emulsions in the presence of NaCl could be attributed to competition by the cations for specific binding sites on phosphoserine groups of caseins, thereby reducing any destabilizing specific effects of Ca^{2+}. This is in accordance with the known decrease in Ca^{2+} binding to both α_{s1}-casein (Dalgleish and Parker, 1980) and β-casein (Parker and Dalgleish, 1981). The strength of Ca^{2+} binding is inversely related to the ionic strength as dictated by the molarity of NaCl.

Binding of Ca^{2+} to β-lg is attributed to electrostatic reactions with ionic amino acid groups of the polypeptide chains, i.e., aspartic and glutamic acids (Zittle et al., 1957; Baumy and Brulé, 1988), and it is weak compared to other milk proteins (Patocka and Jelen, 1991; Pappas and Rothwell, 1991). Fewer binding sites would be available which could be saturated with fewer Ca ions. The presence of NaCl resulted in a marked decrease in the average size of Ca^{2+}-induced β-lg emulsion aggregates (Fig. 2). Baumy and Brulé (1988) observed that when NaCl was present in the buffer, Na^+ may bind to whey proteins in solution in place of other metal cations. Patocka and Jelen (1991) also reported the possibility of Na^+ competing with Ca^{2+} for negative groups as well as for any specific metal binding sites of polypeptide chains in solutions of β-lg. In addition, at a higher ionic strength, the activity coefficient of Ca^{2+} would be lowered.

Results (Fig. 2 to 4) demonstrate the higher Ca^{2+} affinity and sensitivity of emulsions containing sodium caseinate compared to β-lg, since higher average particle sizes were generally obtained at considerably lower concentrations of Ca^{2+}. The bimodal distribution of caseinate emulsion aggregates signifies large cluster formation with strong association. The pattern of aggregation as shown by microscopy supported these observations (Fig. 6). Since caseins bind more Ca^{2+} than β-lg, possibly there were greater potentials for formation of Ca bridges between emulsion particles, in addition to the more general charge effects.

Caseinate emulsions formed visible precipitates at Ca^{2+} concentrations between 7 and 16 mM (Fig. 5). The trend was generally in accordance with other results. However, an important

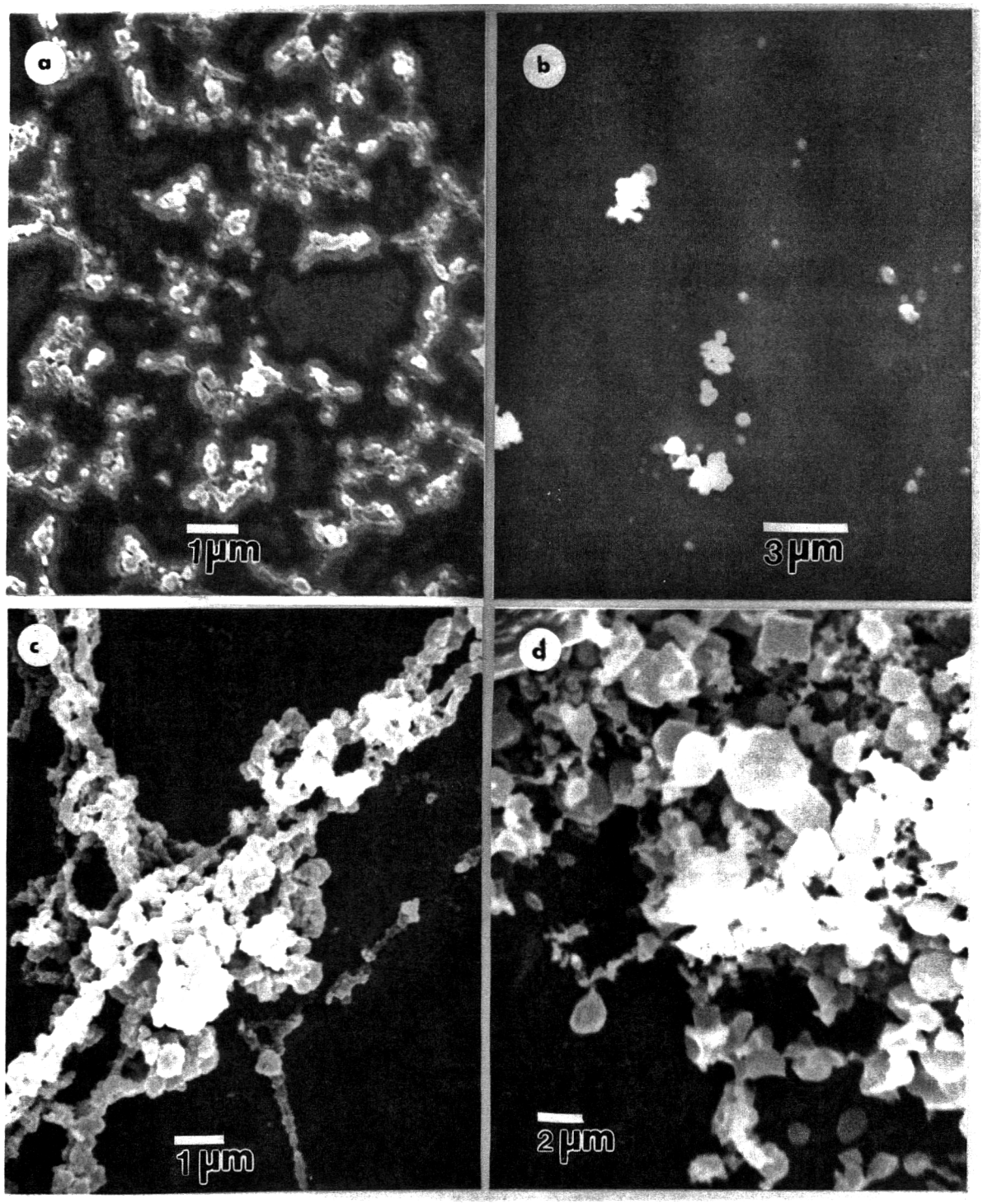

Fig. 6—Cryo-SEM micrographs of emulsions which were stable and destabilized by the presence of 10 mM $CaCl_2$. (a) stable β-lg; (b) stable caseinate; (c) destabilized β-lg; (d) destabilized caseinate.

deviation was the increased stability of emulsions formed with 0.3% protein compared to those formed with 0.5%. This could be due to different conformations of caseins at the oil-water interface as a result of less than optimal protein concentrations (Fang and Dalgleish, 1993). This could lead to changes in overall charge of emulsion droplets and structures of adsorbed proteins.

CONCLUSIONS

STABILITY OF EMULSIONS formed with β-lg and sodium caseinate in the presence of Ca^{2+} depends on the amount of protein as well as the amount of Ca^{2+}. However, the extent of aggregation could largely be associated with the extent of binding of Ca^{2+} by each protein in aqueous solution. The effects of Ca^{2+} in destabilizing emulsions could be reduced by the presence of Na^{+}. Light scattering and microscopic examinations are complementary for the study of the mechanism of the Ca^{2+}-induced aggregation of emulsions stabilized by milk proteins.

REFERENCES

Baumy, J.J. and Brulé, G. 1988. Binding of bivalent cations to α-lactalbumin and β-lactoglobulin: effect of pH and ionic strength. Le Lait 68: 33–48.
Britten, M. and Giroux, H.J. 1991. Coalescence index of protein-stabilized emulsions. J. Food Sci. 56: 792–795.
Dalgleish, D.G. and Parker, T.G. 1980. Binding of Ca ions to bovine α_{s1}-casein and precipitability of the protein-Ca complexes. J. Dairy Res. 47: 113–122.

Dalgleish, D.G., Dickinson, E., and Whyman, R.H. 1985. Ionic strength effects on the electrophoretic mobility of casein-coated polystyrene latex particles. J. Colloid Interf. Sci. 108: 174–179.
Darling, D.F. and Birkett, R.J. 1987. Food colloids in practice. In *Food Emulsions and Foams*, E. Dickinson (Ed.), p. 1–29. Royal Society of Chemistry, London.
de Wit, J.N. and Klarenbeek, G. 1984. Effects of various heat treatments on structure and solubility of proteins. J. Dairy Sci. 67: 2701–2710.
Dickinson, E. and Stainsby, G. 1982. *Colloids in Food*. Applied Science Publishers, London.
Dickinson, E. and Stainsby, G. (Ed.) 1987. *Advances in Food Emulsions and Foams*. Elsevier Applied Science, London.
Dickinson, E., Whyman, R.H., and Dalgleish, D.G. 1987. Colloidal properties of model oil-in-water emulsions stabilized separately by α_{s1}-casein, β-casein and κ-casein. In *Food Emulsions and Foams*, E. Dickinson (Ed.), p. 40–51. Royal Society of Chemistry, London.
Dickinson, E., Hunt, J.A., and Horne, D.S. 1992. Calcium-induced flocculation of emulsions containing adsorbed β-casein or phosvitin. Food Hydrocoll. 6: 359–370.
Emscope Laboratories Ltd. 1982. An evaluation study of the Emscope SP2000 series "Sputter-cryo" low temperature SEM preparation system. Emscope Laboratories Ltd., Ashford, England.
Fang, Y. and Dalgleish, D.G. 1993. Dimensions of adsorbed layers in oil-in-water emulsions stabilized by caseins. J. Colloid Interf. Sci. 156: 329–334.
Horne, D.S. and Dalgleish, D.G. 1980. Electrostatic interaction and the kinetics of protein aggregation: α_{s1}-casein. Int. J. Biol. Macromol. 2: 154–160.
Marshall, R.J. and Green, M.L. 1980. The effect of chemical structure of additives on the coagulation of casein micelle suspensions by rennet. J. Dairy Res. 47: 359–369.
Mulvihill, D.M. 1992. Production, functional properties and utilization of milk protein products. In *Advanced Dairy Chemistry, Vol. 1, Proteins*, P.F. Fox (Ed.), p. 369–404. Elsevier Aplied Science, London and New York.
Mulvihill, D.M. and Kinsella, J.E. 1988. Gelation of β-lactoglobulin: effects of sodium chloride and calcium chloride on the rheological and structural properties of gels. J. Food Sci. 53: 231–236.
Pappas, C.P. and Rothwell, J. 1991. The effects of heating, alone or in the presence of calcium or lactose, on calcium binding to milk proteins. Food Chem. 42: 183–201.
Parker, N.S. 1988. Properties and functions of stabilizing agents in food emulsions. CRC Critical Reviews in Food Science & Nutrition 25: 285–315.
Parker, T.G. and Dalgleish, D.G. 1981. Binding of Ca ions to bovine β-caseins. J. Dairy Res. 48: 71–76.
Patocka, G. and Jelen, P. 1991. Ca association with isolated whey proteins. Can. Inst. Food Sci. Technol. J. 24: 218–223.
Payens, T.A.J. 1979. Casein micelles: the colloid chemical approach. J. Dairy Res. 46: 291–306.
Swaisgood, H.E. 1992. Chemistry of the caseins. In *Advanced Dairy Chemistry, Vol. 1, Proteins*, P.F. Fox (Ed.), p. 63–110. Elsevier Aplied Science, London and New York.
Tornberg, E. 1978a. Functional characterization of protein-stabilized emulsions: creaming stability. J. Food Sci. 43: 1559–1565.
Tornberg, E. 1978b. Functional characterization of protein-stabilized emulsions: Emulsifying behaviour of proteins in a valve homogenizer. J. Sci. Food. Agric. 22: 867–879.
Vincent, B. 1974. The effects of adsorbed polymers on dispersion stability. Adv. Colloid Interf. Sci. 4: 196–277.
Zittle, C.A., Dellamonica, E.S., Rudd, R.K., and Custer, J.H. 1957. The binding of calcium ions by β-lactoglobulin before and after aggregation by heating in the presence of calcium ions. J. Amer. Chem. Soc. 79: 4661–4666.
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Free Amino Acid Stability in Reducing Sugar Systems

JEFFREY H. BAXTER

ABSTRACT

Solutions of free amino acids were sterilized in different carbohydrate mixtures, at different pH values, and both browning and free amino acid content monitored at different storage temperatures. Results showed the expected behavior of a Maillard process, (e.g., increasing reaction rate with increases in pH, temperature and reducing sugar content). However, only three (tryptophan, histidine and lysine) of the amino acids tested (nine essential + taurine) were notably affected, even under stringent conditions (50°C storage, glucose containing solution at pH 7.5). Thus many free amino acids could probably be added to most food matrices without Maillard reactions affecting overall protein quality.

Key Words: amino acids, Maillard browning, protein stability, reducing sugars.

INTRODUCTION

REACTIONS SUCH AS MAILLARD BROWNING cause problems with product appearance (brown color with age) and protein quality, especially available lysine (for reviews, see Kaanane, 1989; Mauron, 1990; Namiki, 1988; Waller and Feather, 1983). There are other reasons for concern in the food industry. For free amino acids, methionine (Baker et al., 1984), lysine (c.f.: Sherr et al., 1989), and other amino acid Maillard products (c.f.: Desrosiers and Savoie, 1991) are not bioavailable. The report that peptides containing N-terminal methionine were susceptible to Maillard-linked loss of bioavailable methionine (Baker et al., 1984) indicates that amino acid nutrition may be diminished as a result of these reactions, even if small peptides were present (e.g., in some hydrolysates) instead of free amino acids. Some products from Maillard reactions have reduced digestibility of protein sources (Oste, 1989, 1991). The formation of low molecular weight inhibitors of gastrointestinal proteolytic digestive enzymes has been shown (Oste et al., 1986; Igarashi et al., 1983). This indicated that Maillard reaction products also prevented the efficient digestion of peptides and proteins in general. Advanced Maillard products have also been reported to be mutagenic (c.f.: Hiramoto et al., 1993; Skog, 1993), anti-mutagenic (Yen et al., 1992), toxic (O'Brien and Morrissey, 1989) and to affect mineral bioavailability (Andrieux and Sacquet, 1984; Whitelaw and Weaver, 1988). These results have demonstrated the wide range of problems associated with Maillard reactions.

Based on experience as well as published data, many processors design food products to minimize the extent of such reactions, and seek to assure protein quality by analyzing for available lysine. In products containing only intact protein this approach has been adequate, though losses of some non-protein associated amino acids (e.g., taurine) have been observed in food products in our laboratory. In products with a substantial content of either hydrolyzed protein or free amino acids, severe restrictions of the type carbohydrate, and use of acidic pH have been applied. One study reported inhibition of the overall browning process by addition of acidic amino acids (Nafisi and Markakis, 1983), suggesting that other, less traditional modifications of food composition might be advantageous.

Many publications are available concerning the Maillard reactions (for review, c.f., Erbersdobler, 1989). Most studies have focussed on mixtures of reactants in relatively low moisture (10–20% by weight), and relatively short time spans under high heat (Saltmarch and Labuza, 1982; Labuza and Saltmarch, 1981). The extent of reaction has been substantial, often consuming more than 50% of some of the amino acids initially present. Neither the extent nor rate of reactions of free amino acids in relatively dilute aqueous solutions (containing notable amounts of reducing sugars) has been extensively studied. Data suggest that such reactions occur. Several groups noted considerable differences in susceptibility of amino acids to such reactions in model systems heated intensely for short times (c.f., Ames, 1986; Ashoor and Zent, 1984; Gothwal and Bhavdasan, 1991; Obretenov et al., 1990). One group (Labuza and Massaro, 1990) addressed the kinetics of lysine, cysteine and tryptophan loss (in a model parenteral nutrition system aged at 4 or 30°C) but other essential amino acids were not studied. The growing use of protein hydrolysates in the food industry, coupled with more stringent dietary regulations indicates that a study is needed to define the potential extent of losses of essential amino acids to such reactions.

The objective of this study was to determine the relative stability of essential amino acids under conditions similar to those in liquid, protein-containing nutritional products.

MATERIAL & METHODS

UNLESS OTHERWISE INDICATED, all chemicals were from Sigma Chemical Co. (St. Louis, MO).

Experimental mixture

Lysine monohydrochloride (30.0 g), valine (20.4 g), threonine (20.8 g), leucine (22.8 g), histidine (27.0 g), taurine (21.8 g), tryptophan (35.5 g), isoleucine (22.8 g), phenylalanine (28.7 g) and methionine (26.0 g) were all dissolved in 9.7 kg of water (Milli-Q) by paddle agitation. Carbohydrate (glucose, sucrose, maltose, lactose or fructose) was added (1 kg total) and the solution stirred until all solids were dissolved. If carbohydrate was not added, additional water to compensate for mass difference was added. The solution was adjusted to the target pH (5.5, 6.5, or 7.5) with either 6*N* HCl or 8*N* NaOH, weighed (270 ± 5 g) into metal cans (280 mL zinc coated two-piece cans used for nutritional products) and hermetically sealed. The test mixtures were retort sterilized using a commercial process with a 225 sec hold at 128°C. After cooling, samples were stored (5°C, 37.5°C or 50°C) until analysis. Samples were withdrawn for testing prior to and immediately after sterilization, as well as after storage for up to 126 wk (one can per time/temperature point). Prior to sterilization all test mixtures had negligible absorbance (<0.05). During this study, no changes were observed in the interior surface of the can.

Absorbance measurements

Absorbance at 420 nm (c.f., Baisier and Labuza, 1992) was measured using a Beckman DU-30 Spectrophotometer. A linear range up to 1.0 AU was established. If the sample absorbance exceeded that value, a suitable dilution into water was made to enable measurement within the linear range. All measurements were in duplicate, once an appropriate dilution factor was selected. Values were adjusted for dilution factors.

Free amino acid analysis

Amino acid analysis was by reversed phase HPLC separation of sample derivatized (pre-column) with o-phthalaldehyde (OPA) modified

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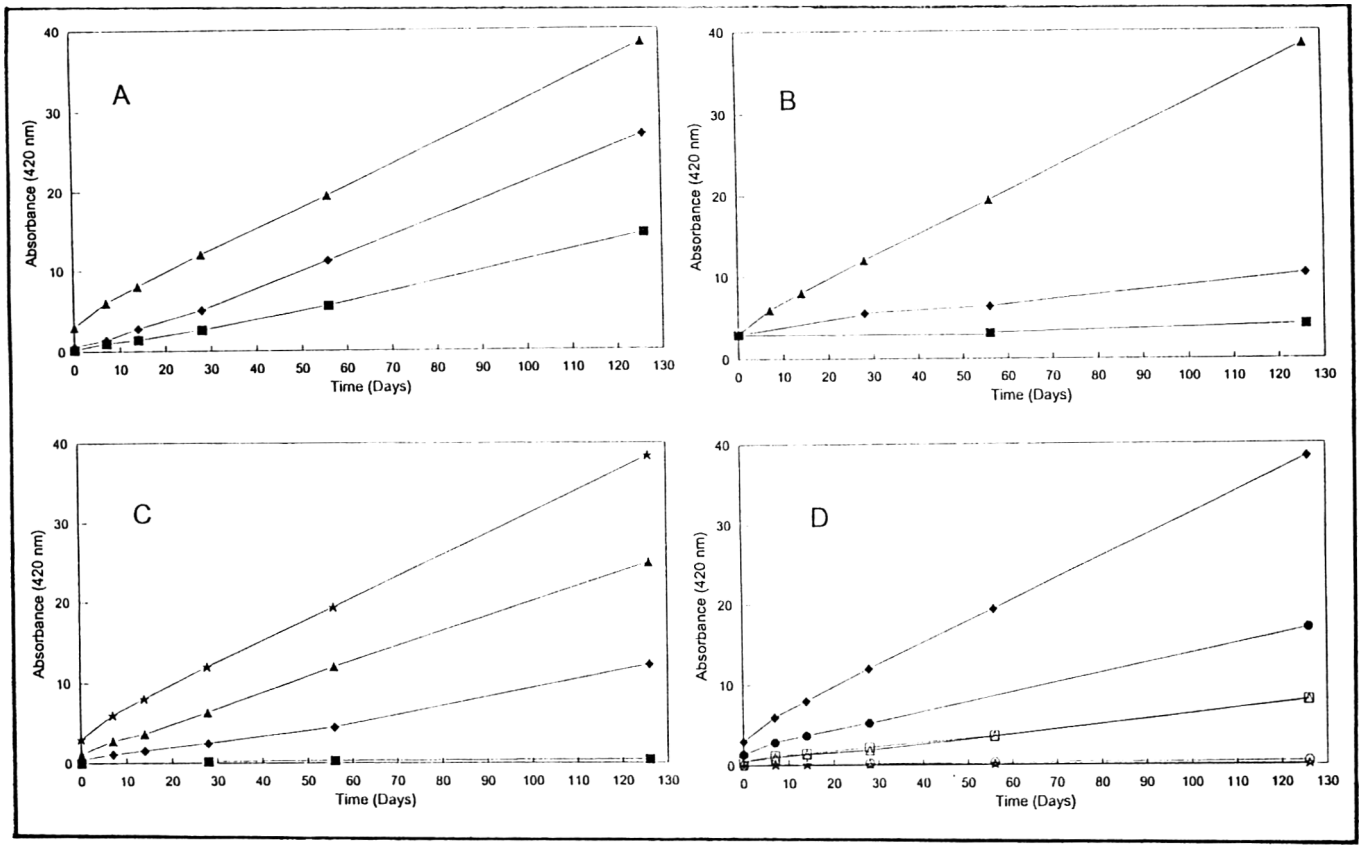

Fig. 1—Absorbance changes during storage. Data at 0-time was after sterilization. (A)—Amino acid solution containing glucose, stored at 50°C and pH 5.5 (■), pH 6.5 (♦) and pH 7.5 (▲). (B)—Amino acid solution containing glucose at pH 7.5, stored at 4°C (■), 38.5°C (♦) and 50°C (▲). (C)—Amino acid solution at pH 7.5, stored at 50°C and containing 9.13% (w/w) sucrose (■), 6.39% (w/w) sucrose and 2.74% (w/w) glucose (♦), 2.74% (w/w) sucrose and 6.39% (w/w) glucose (▲) or 9.13% (w/w) glucose (★). (D)—Amino acid solution at pH 7.5 stored at 50°C and containing no sugar (☆) or 9.13% (w/w) sucrose (○), lactose (△), maltose (□), fructose (●) or glucose (♦).

from Jones et al. (1981) by using 3-mercaptopropionic acid (3-MPA) as suggested by Kucera and Umgat (1983). Chromatography was optimized for the simpler test mixture, and internal standard correction and external standard calibration were used to determine concentrations from peak areas. Using this approach, the relative standard deviation for duplicate analyses was ≤3.5% for any analyte throughout the experiment.

Derivatizing solution

OPA Reagent (10 mL) Incomplete (Sigma Chemical Co., St. Louis, MO) was mixed with 100 µL 3-MPA immediately prior to use.

Sample preparation

Test solution (0.5 mL) was mixed with 1 mL of internal standard solution (1 g/L α-amino n-butyric acid in water) and diluted to 100 mL final volume with Milli-Q water. An aliquot of this solution (200 µL) was added to 500 µL of derivatizing solution in a clean amber vial, mixed and allowed to incubate for exactly 1.7 min prior to injection. This analysis was done in duplicate for each time/temperature point, and mean values were reported.

HPLC system

Column—APEX II C-18, 4.6 mm × 15 cm, 3 micron (Jones Chromatography, Littleton, CO). Mobile Phase A—1% (v/v) glacial acetic acid (Mallinckrodt, Inc., Paris, KY) 0.4% (v/v) triethylamine, pH 5.0. Mobile Phase B—Mix 1 volume methanol and 1 volume acetonitrile (Burdick and Jackson, Muskegon, MI). After injecting 20 µL of derivitized sample (or standards), a gradient of 15% B to 50% B in 20 min followed by 50% B to 100% B in 5 min and a 5 min hold eluted all analytes. Column temperature was 40°C and a 1 mL/min flow rate was used. Eluant was monitored using a Shimadzu RF 535 fluorescence detector with excitation at 330 nm and emission at 450 nm. Quantitation was by peak area comparison with standard amino acids.

Effect of acid hydrolysis

With or without borohydride reduction—0.5 mL of test solution was placed in each of 2 50mL ampules, and 0.11g sodium borohydride was added to one ampule ("Available" AA determination; Couch and Thomas, 1976). These ampules were allowed to stand for 1 hr. 1 mL of internal standard (see above) and 20 mL of 6N HCl were added to both ampules, and they were flushed with nitrogen and flame sealed. After incubation for 22 hr at 110°C, the ampules were cooled to room temperature (≈23°C) opened, and the contents diluted to ≈50 mL by addition of Milli-Q water. After mixing thoroughly, the contents were filtered through glass fiber filters into 500 mL round bottom flasks, and evaporated to dryness using a rotary evaporator. The solid was dissolved in water, transferred to 100 mL volumetric flasks and diluted to final volume with water. After mixing, amino acid analysis was performed as described. The sodium borohydride treatment did not notably alter the chromatography (data not shown).

RESULTS & DISCUSSION

BROWNING REACTIONS, previously studied extensively in powders, showed similar dependence on pH, (Fig. 1A) temperature (Fig. 1B) and reducing sugar (Fig. 1C) in these liquid systems; i.e., browning increased as pH, temperature and reducing sugar content increased. As expected, glucose (an aldohexose) was very reactive, while fructose (a ketohexose) was less reactive and almost indistinguishable from the disaccharides, though it theoretically has two times the reducing equivalents. The reducing disaccharides lactose and maltose were similar in reactivity, while a nonreducing disaccharide, sucrose was essentially inert (Fig. 1D). A small amount of reducing sugar was enough to cause considerable browning, and loss of susceptible free amino acids under stringent conditions. As with powders, adjusting pH is an effective method to reduce browning, and controlled tem-

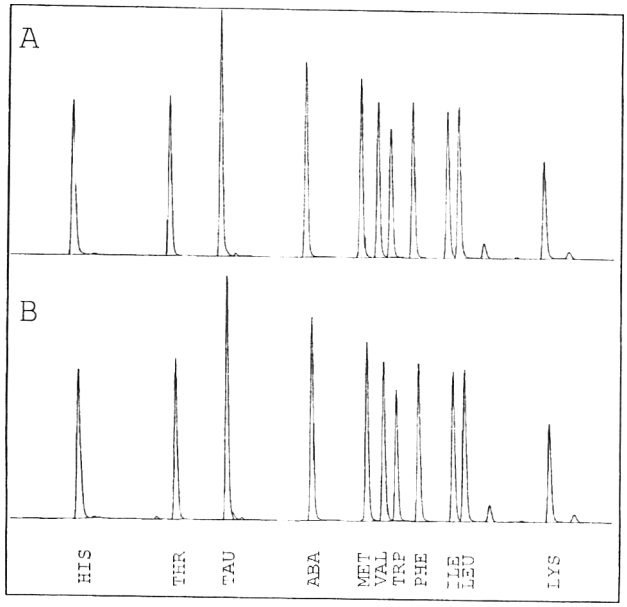

Fig. 2—Amino acid analysis chromatography. (A) Amino acids standards mixture. (B) Amino acids mixture containing 9.13% (w/w) fructose, pH 7.5, stored 7 wk at 50°C. Free amino acid analysis (no sodium borohydride treatment) as described in Materials and Methods. Relative fluorescence intensity is recorded versus elution time (0—30 min). Amino acids are indicated by the standard three letter abbreviations.

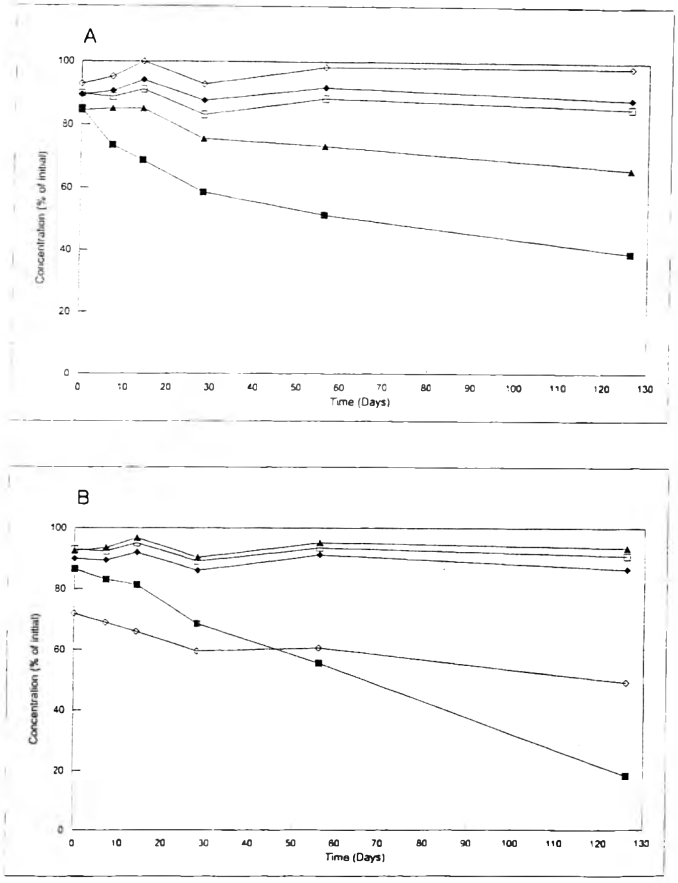

Fig. 3—Amino acid losses vs time of storage. Solutions containing amino acids and 9.13% (w/w) glucose at pH 7.5 were sterilized and stored at 50°C. Data are presented as percent of the nonsterilized 0-time analytical result. Data plotted as 0-time are immediately post sterlization, without storage. (A)—valine (◇), threonine (♦), methionine (□), taurine (▲) and histidine (■). (B)—isoleucine (▲), leucine (□), phenylalanine (♦), lysine (◇) and tryptophan (■).

perature storage is also helpful. Beyond this, careful selection of carbohydrate type could also lessen browning.

The amino acid analytical method provided baseline resolution of all amino acids in the test mixture (Fig. 2A). Incubation of the amino acids with test sugars, while inducing decreases in some of amino acid peaks, did not generate new, interfering peaks (Fig. 2B).

The initial reaction of the Maillard process involves formation of a Schiff's base by the sugar and the amino acid. This reaction is reversible. However, subsequent rearrangements are not reversible, and result in "destruction" of the amino acid. A Schiff's base is labile to acid, and acid hydrolysis followed by amino acid analysis gives free + Schiff's base amino acid. However, reduction by sodium borohydride would produce the secondary amine from a Schiff's base. The secondary amine is not acid labile, and acid hydrolysis after such reduction, followed by amino acid analysis would only detect free amino acids (Couch and Thomas, 1976). Using this approach, we analyzed several aged samples where substantial browning and amino acid losses had occurred. No evidence for notable "trapping" in the Schiff's base was observed (data not shown). Apparently, initial reaction products, once formed, were subject to rapid subsequent Maillard process rearrangements, leading to "loss" of the amino aids.

Amino acid analysis of aged samples showed rather sharp differences in reactivities of the amino acids tested. To illustrate this, data for one system (pH 7.5, solution containing glucose, stored at 50°C) are presented (Fig. 3). All the amino acids tested showed some losses upon retort sterilization. Losses ranged from 8–14% for all except lysine which was exceptionally sensitive (28% loss). However, after sterilization, only tryptophan, histidine, lysine, and taurine showed further significant loss during storage. While lysine was quite sensitive (total loss of 61% after 126 wk of storage), both histidine (61% loss) and tryptophan (82% loss) were as, or more, sensitive over the same time period. Losses of these amino acids, however may involve other reactions besides Maillard browning. Note that no significant losses were observed for threonine, valine, methionine, phenylalanine, isoleucine or leucine, even under such stringent conditions. Also these amino acids showed lower processing losses (7–11%) than the susceptible compounds (13–28%).

To illustrate the effect of various parameters known to affect Maillard processes, the data for one of the sensitive amino acids, histidine, are presented (Fig. 4). The loss of histidine appeared to follow all trends generally associated with a Maillard-type process. Increasing pH (Fig. 4A), temperature (Fig. 4B) and reducing sugar content (Fig. 4C) resulted in higher losses over time. Glucose was the most reactive carbohydrate resulting in ≈61% loss (Fig. 4D). Fructose was much less reactive, with about 32% loss of histidine after 126 wk storage. Maltose and lactose were essentially equivalent, with losses of ≈20%, while the sample with sucrose was indistinguishable from control (no sugar). All of these observations were consistent with Maillard-type reactions.

These data demonstrated that the chemical differences between the amino acids resulted in a wide range of susceptibility to the Maillard process. Not all amino acids were labile to losses via Maillard reactions to nearly the same extent. This information is useful for formulation of food products, especially considering the interest in more hydrolyzed proteins in special applications. There are advantages to hydrolysates, regarding digestibility and tolerance or hypoallergenicity. The hindrance to their applicability has always been the high browning potential, with its associated problems. Also, some relatively low cost or otherwise desirable protein systems have not been used because fortification with amino acids would be required to ensure nutritional adequacy, and these have been presumed to be Maillard labile.

The data from these experiments indicate that such presumptions are not necessarily true. Some of the essential amino acids

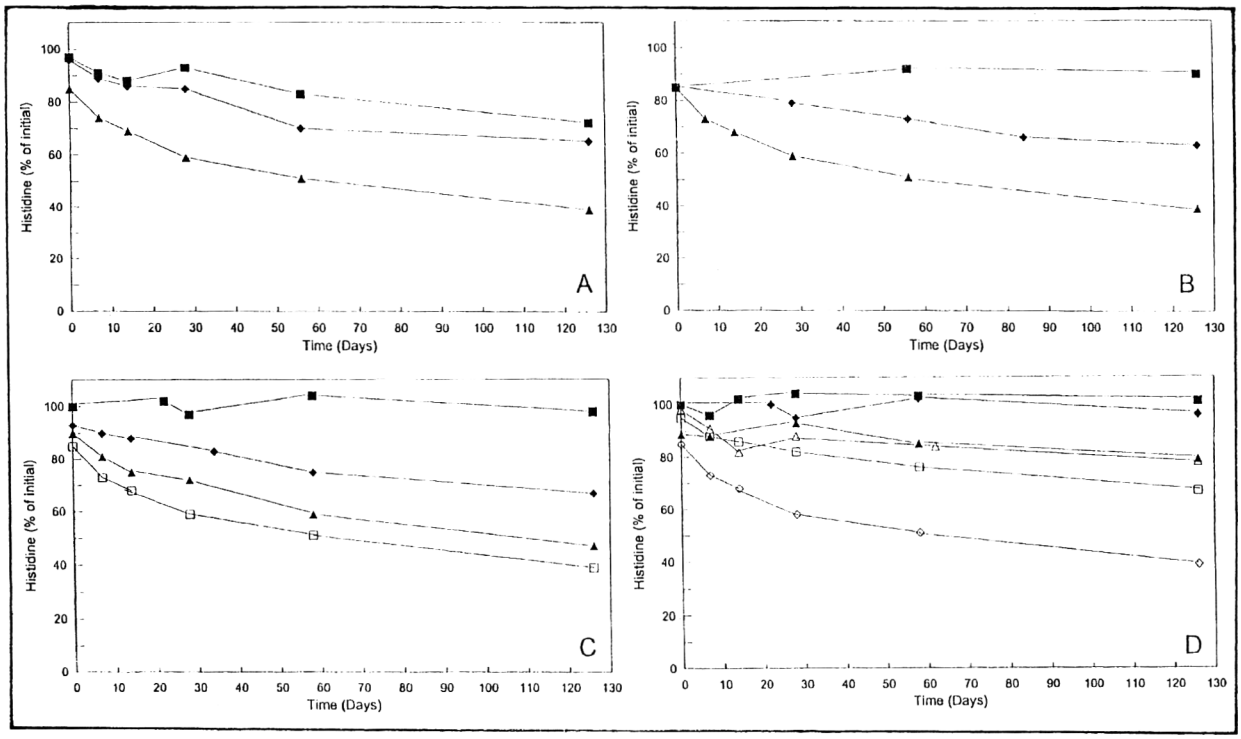

Fig. 4—Relation of histidine loss to test parameters. Data are plotted as percent of the nonsterilized 0-time analysis result. Data plotted at 0-time are from a solution which was sterilized but not yet stored. (A)—Dependance on solution pH (9.13% (w/w) glucose solution stored at 50°C) pH 5.5 (■), 6.5 (♦) or 7.5 (▲). (B)—Dependance on storage temperature (pH 7.5, 9.13% (w/w) glucose), 4°C (■), 38.5°C (♦) or 50°C (▲). (C)—Dependance on reducing sugar content (pH 7.5, 50°C storage) and 9.13% (w/w) sucrose (■), 6.39% (w/w) sucrose + 2.74% (w/w) glucose (♦), 2.74% (w/w) sucrose + 6.39% (w/w) glucose (▲) or 9.13% (w/w) glucose (□). (D)—Dependance on carbohydrate type (pH 7.5, stored at 50°C and containing (if added) 9.13% (w/w) carbohydrate), no carbohydrate (■), sucrose (♦), maltose (△), fructose (□), lactose (▲) or glucose (◇).

could be fortified without special storage or severe limitations on carbohydrate source. This may provide opportunities for product development. However, potential effects of low levels of advanced Maillard reaction products must be considered.

REFERENCES

Ames, J.M. 1986. A comparison of the browning potential of several food-related amino acids heated with D-(+)-xylose at neutral pH. Chem. Ind. 10: 362–363.

Andrieux, C. and Sacquet, E. 1984. Effects of Maillard's reaction products on apparent mineral absorption in different parts of the digestive tract. The role of microflora. Reprod. Nutr. Develop. 24(4): 379–386.

Ashoor, S.H. and Zent, J.B. 1984. Maillard browning of common amino acids and sugars. J. Food Sci. 49: 1206–1207.

Baisier, W.M. and Labuza, T.P. 1992. Maillard browning kinetics in a liquid model system. J. Agric. Food Chem. 40(5): 707–713.

Baker, D.H., Bafund, K.W., Boebel, K.P., Czarnecki, G.L., and Halpin, K.M. 1984. Methionine peptides as potential food supplements: Efficacy and susceptibility to Maillard browning. J. Nutr. 114: 292–297.

Couch, J.R. and Thomas, M.C. 1976. A comparison of chemical methods for the determination of available lysine in various proteins. J. Agric. Food Chem. 24(5): 943–946.

Desrosiers, T. and Savoie, L. 1991. Extent of damage to amino acid availability of whey protein heated with sugar. J. Dairy Res. 58: 431–441.

Erbersdobler, H.F. 1989. Protein reactions during food processing and storage—Their relevance to human nutrition. Bibl. Nutr. Dieta. 43: 140–155.

Gothwal, P.P. and Bhavdasan, M.K. 1991. Studies on browning reaction between common amino acids and sugars. Asian J. Dairy Res. 10(4): 199–204.

Hiramoto, K., Kato, T., and Kikugawa, K. 1993. Generation of DNA-breaking activity in the Maillard reaction of glucose-amino acid mixtures in a solid system. Mutation Res. 285: 191–198.

Igarashi, K., Tsunekuni, T., and Yasui, T. 1983. Inhibition of proteolytic activity of papain by browning reaction products of quercetin. J. Nutri. Sci. Vitaminol. 29: 227–232.

Jones, B.N., Svante, P., and Stein, S. 1981. Amino acid analysis and enzymatic sequence determination of peptides by an improved o-phthaldialdehyde precolumn labeling procedure. J. Liq. Chrom. 4: 565–586.

Kaanane, A. and Labuza, T.P. 1989. The Maillard reaction in foods. Prog. Clin. Bio. Res. 304: 301–327.

Kucera, P. and Umagat, H. 1983. Design of a post-column fluorescence derivitization system for use with microbore columns. J. Chrom. 255: 563–579.

Labuza, T.P. and Massaro, S.A. 1990. Browning and amino acid loss in model total parenteral nutrition solutions. J. Food Sci. 55(3): 821–826.

Labuza, T.P. and Saltmarch, M. 1981. The non-enzymatic browning reaction as affected by water in foods. In *Water Activity: Influences on Food Quality*, L.B. Rockland and G.F. Stewart (Ed.), p. 605–650. Academic Press, New York.

Mauron, J. 1990. Influence of processing on protein quality. J. Nutr. Sci. Vitaminol. 36: S 57–S 69.

Nafisi, K. and Markakis, P. 1983. Inhibition of sugar-amine browning by aspartic and glutamic acids. J. Agric. Food Chem. 31: 1115–1117.

Namiki, M. 1988. Chemistry of Maillard reactions: Recent studies on the browning reaction mechanism and the development of antioxidents and mutagens. Adv. Food Res. 32: 115–184.

O'Brien, J. and Morrissey, P.A. 1989. Nutrional and toxicological aspects of the Maillard browning reaction in foods. Critical Rev. Food Sci. Nutr. 28(3): 211–342.

Obretenov, T.D., Kuntcheva, M.J., and Panchev, I.P. 1990. Influence of the reaction conditions on the formation of non-dialyzable melanoidines from D-glucose and L-amino acids. J. Food Processing Preservation 14: 309–324.

Oste, R.E. 1989. Effects of Maillard reaction products on protein digestion. Prog. Clin. Biol. Res. 304: 329–342.

Oste, R.E. 1991. Digestibility of processed food protein. Adv. Exp. Med. Biol. 289: 371–388.

Oste, R.E., Dahlquist, A., Sjostrom, H., Noren, O., and Miller, R. 1986. Effect of Maillard reaction products on protein digestion. *In vitro* studies. J. Agric. Food Chem. 34: 355–358.

Sherr, B., Lee, C.M., and Jelesciewicz, C. 1989. Absorption and metabolism of lysine Maillard products in relation to utilization of L-lysine. J. Agric. Food Chem. 37: 119–122.

Saltmarch, M. and Labuza, T.P. 1982. Non-enzymatic browning via the Maillard reaction in foods. Diabetes 31: 29–36.

Skog, K. 1993. Cooking procedures and food mutagens: A literature review. Fd. Chem. Toxic. 31: 655–675.

Waller, G.R. and Feather, M.S. (Ed.). 1983. *The Maillard Reaction in Foods and Nutrition*. M.J. Comstock (Ed.). ACS Symposium Series, American Chemical Society, Washington, DC.

Whitelaw, M.L. and Weaver, C.M. 1988. Maillard browning effects on *in vitro* availability of zinc. J. Food Sci. 53(5): 1508–1510.

Yen, G.-C., Tsai, L.-C., and Lii, J.-D. 1992. Antimutagenic effect of Maillard browning products obtained from amino acids and sugars. Fd. Chem. Toxic. 30(2): 127–132.

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Direct ELISAs for Sulfathiazole in Milk and Honey with Special Emphasis on Enzyme Conjugate Preparation

CARRIE A. THOMSON and PETER SPORNS

ABSTRACT

Direct competitive enzyme-linked immunosorbent assays (ELISAs) were developed for sulfathiazole (ST) analysis. Polyclonal antibody rabbit serum adsorption to polystyrene microtiter plates was enhanced using a 1 µg/mL Protein A precoating solution. Detection limits (20% reduction in maximum enzyme signal) for ST in milk and honey were 12.0 and 34.7 ppb (ng/L), respectively. ST-horseradish peroxidase (HRP) conjugates prepared by linking the N^4-amino group of ST to periodate-treated HRP were more efficient than those prepared using a more conventional two-step glutaraldehyde method. This represents a new method of preparing sulfonamide conjugates for use in ELISAs.

Key Words: polyclonal, antibody, horseradish peroxidase, milk, honey, sulfathiazole

INTRODUCTION

SULFONAMIDES (SAs) are synthetic derivatives of sulfanilic acid which exert antimicrobial activity (Hays, 1986). In animal husbandry, SAs are used to treat sick animals, to increase feed efficiency, or as a prophylactic measure (Hays, 1986; Kaneene and Miller, 1992). In apiculture, sulfathiazole (ST) is effective against American foulbrood, a devastating infection of honeybee larvae (Argauer, 1986; Eckert, 1947; Haseman, 1953).

Once administered to livestock, SAs are distributed throughout body tissues (Anand, 1975), which can lead to unlawful residue levels in milk and meat derived from SA-treated animals if withdrawal times are not adequate (Huber, 1986). Feeding of ST to honeybee colonies can similarly lead to contamination of the honey crop. While negligible degradation occurs in honey (Belliardo, 1981; Horie et al., 1992), some reaction with honey sugars has been demonstrated (Sheth et al., 1990).

Concern over SA residues in foods has arisen for legal, processing, and public health reasons. SA residues in milk can interfere with dairy starter cultures (Schiffmann et al., 1992). Public health concerns include the possibility that target microorganisms may develop SA resistance and such strains may be transmitted to humans (Van Poucke et al., 1991). There is also potential for idiosyncratic allergic reactions in ultrasensitive consumers (Brady and Katz, 1988; Cribb et al., 1991), and alleged carcinogenicity of certain SAs (Woodward, 1991, 1992).

Legal tolerance levels for sulfonamides are continuously reevaluated as new toxicological information becomes available (Long et al., 1990). In the U.S., the FDA has established 10 ppb as the safe level for all sulfonamides (Zomer et al., 1992). Current Canadian regulations stipulate maximum residue levels (of 10 ppb) of the SA sulfadimethoxine in milk (Canadian Gazette, 1991). While Canadian regulations specify zero tolerance for ST residues in honey (Neidert et al., 1986), the enforcement level is set at 200 ppb due to difficulties encountered in routinely assaying at lower levels (Stecyk, 1987).

Many different analytical techniques are available for SAs. Chromatographic methods have been applied (Abián et al., 1993; Agarwal, 1992; Argauer et al., 1982; Barry and MacEachern, 1983; Horie et al., 1992; Jürgens, 1982; Larocque et al., 1990; Parks, 1982; Roudaut and Moretain, 1990; Takeda and Akiyama, 1991, 1992; Van Poucke et al., 1991; Weber and Smedley, 1983; Zomer et al., 1992), but often have low recoveries and reproducibility (Horwitz, 1981), and require extraction and extensive clean-up procedures (Agarwal, 1992; Horwitz, 1981). Furthermore, detection limits of reverse-phase chromatographic methods are usually in the order of 1000 ppb (Belliardo, 1981; Diaz et al., 1990; Jürgens, 1982), which is outside the range required. Microbiological assays are time-consuming and quantify only the biologically active form of SAs, and not metabolites (Katz, 1986). Colorimetric methods can provide low detection limits (25–100 ppb) (Schwartz and Sherma, 1986); however, they tend to be less-specific and do not quantify individual sulfonamides (Horwitz, 1981).

Immunological methods permit rapid and sensitive means of detecting SAs at current legal limits (Assil et al., 1992; Dixon-Holland and Katz, 1988, 1989, 1991; Fleeker and Lovett, 1985; Hoffmeister et al., 1991; McCaughey et al., 1990; Sheth and Sporns, 1990, 1991; Sheth et al., 1990; Singh et al., 1989). Our objective was to develop simple direct competitive enzyme-linked immunosorbent assays (ELISAs) for ST in milk and honey.

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MATERIALS & METHODS

Materials

Glutaraldehyde (grade II, 25% aqueous solution), 3,3',5,5'-tetramethylbenzidine dihydrochloride (TMB-d), bovine serum albumin (BSA) (fraction V), horseradish peroxidase (HRP) (Type VI, RZ=3.1), L-lysine, thimerosal, sodium sulfathiazole, sulfathiazole (free acid) and polyoxyethylenesorbitan monolaurate (Tween 20) were obtained from the Sigma Chemical Co. (St. Louis, MO). Sodium carbonate and dimethyl sulfoxide were obtained from Fisher Scientific (Edmonton, AB), and urea peroxide, protein A (PA) (from *Staphylococcus aureus*), and goat anti-rabbit (GαR) IgG (Fc; affinity-purified antibody) were obtained from Calbiochem (San Diego, CA). Sodium borohydride was from Anachemia (Edmonton, AB). All other chemicals were of reagent grade or better. Water was purified using a Millipore Milli-Q ultrafiltration system (Millipore (Canada) Ltd., Mississauga, ON).

An Accumet 925 pH/ion meter was used to measure pH values (Fisher Scientific, Edmonton, AB). Dialysis tubing was Spectra/Por Membrane 1 (MWCO 6,000–8,000) and Membrane 2 (MWCO 12,000–14,000), (Spectrum Medical Industries, Inc., Los Angeles, CA). Immulon 2 polystyrene microtiter plates were from Dynatech Laboratories, Inc. (Chantilly, VA). Microtiter plates were read on an Automated Microplate Reader, Model EL309, (Bio-Tek Instruments, Inc., Burlington, VT). Samples were flash-evaporated using a Büchi Rotavapor RE 121 with a Büchi 461 water bath (Büchi, Switzerland). Linbro acetate adhesive plate sealers were obtained from ICN Biomedicals (Costa Mesa, CA). Competitive inhibition curves from ELISA data were analyzed using *SOFTmax* software (Molecular Devices Corporation, Menlo Park, CA). Waters C_{18} Sep-Pak cartridges were obtained from the Millipore Corporation (Milford, MA). Spectrophotometric measurements were made using an HP8451A diode array spectrophotometer (Hewlett Packard, Canada, Ltd., Mississauga, ON); samples were contained in UV semi-micro cuvettes (Fisher Scientific, Edmonton, AB).

Honey known to be free of ST was obtained from a local Edmonton beekeeper. Samples were diluted in milli-Q water on a w/v basis and filtered by gravity through Whatman No. 1 paper. Selected samples were treated using Waters C_{18} cartridges which were conditioned as per manufacturer's recommendation; eluate was used directly in ELISAs. A

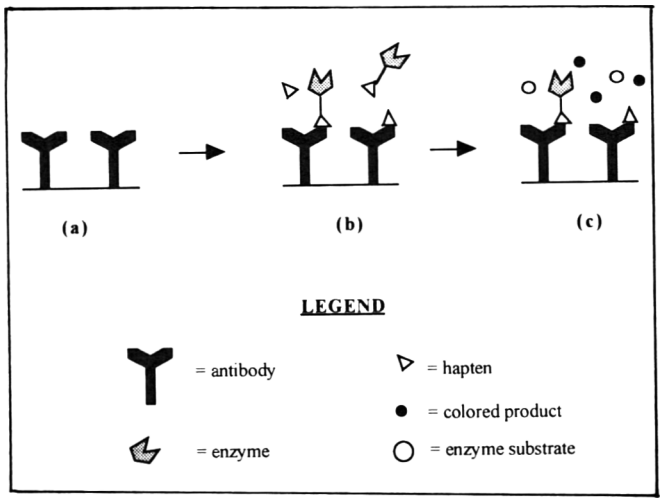

Fig. 1—Direct competitive ELISA. (a) Coat plate with Ab; (b) add free hapten (i.e., standard or sample) and enzyme-conjugated hapten; (c) add enzyme substrate and develop color.

honey analog was prepared by dissolving D-glucose (33.8g) and D-fructose (38.8g) volumetrically in 100 mL milli-Q water and filtering through Whatman No. 1 paper. The pH values of 15-mL aliquots of 1:3 aqueous honey dilutions were adjusted to ≈4, 5, 6, 7, and 8 using 0.05 N or 0.1 N sodium hydroxide. Samples were mixed and allowed to equilibrate overnight and adjusted when necessary to approximate target pH values. Homogenized milk samples (3.5% fat) were obtained from a local supermarket and used undiluted in ELISA protocols.

Anti-sulfathiazole polyclonal antibody serum

Polyclonal serum specific for ST was developed in rabbits as detailed by Sheth et al. (1990).

Synthesis of horseradish peroxidase-sulfathiazole (HRP-ST) conjugates

HRP-ST(G) conjugates were synthesized using the general procedure of Dixon-Holland and Katz (1991) for glutaraldehyde cross-linking. Three HRP-ST(G) conjugates were synthesized using the glutaraldehyde method by varying amounts of ST and HRP. To synthesize HRP-ST(P) conjugates by periodate cleavage, the procedure employed was based on the method of Wilson and Nakane (1978). HRP (4.1 mg) was dissolved in 1.0 mL milli-Q water; 200 µL of 100 mM aqueous sodium periodate were added and the mixture stirred 20 min at room temperature, transferred to dialysis tubing (12,000–14,000 MWCO) and dialyzed overnight (~18 hr) against 1 L of 1 mM sodium acetate buffer (pH 4.4) at 4°C. To dialysis tubing contents were added 20 µL of 200 mM carbonate buffer (pH 9.5) and two drops of 1N NaOH; the reaction mixture was stirred for 2 hr at 4°C. Sodium borohydride (4 mg/mL, 100 µL) was added and the mixture stirred 2 hr at 4°C. The reaction mixture was dialyzed against three changes of PBS (without thimerosal) for a minimum of 3 hr. The contents of the dialysis tubing (~2 mL) were recovered and frozen in 0.5-mL aliquots at −20°C. A total of three conjugates was produced by periodate cleavage by varying the amounts of HRP and ST in the reaction.

Standard direct competitive ELISA protocol

Microtiter plates were pre-coated with 1.0 µg/mL Protein A (100 µL/well) (Schneider and Hammock, 1992), covered and incubated overnight (~18 hr) at 4°C. All further incubations were carried out at room temperature. The next day, the solution was removed from the wells and serum added (200 µL/well); microtiter plates were re-covered and incubated 2 hr. After removal of serum, microtiter plates were blocked using 1% BSA in PBS (200 µL/well) (1 hr incubation). A general washing (3×200 µL/well PBST) was performed and HRP-ST (100 µL/well) diluted in PBST and 100 µL/well of aqueous ST standard were added; this competition step proceeded for 2 hr. After a final general washing, TMB-d substrate solution (see below) was added (200 µL/well) and color allowed to develop at room temperature (Fig. 1). The reaction was stopped by adding 50 µL/well 2 M sulfuric acid; absorbance values were read at 450 nm. TMB-d (10 mg) was dissolved in 1.0 mL dimethyl sulfoxide and added to 100 mL of 0.1 M citrate buffer (pH 4.0) containing 100 mg urea peroxide.

Precoating with goat-anti-rabbit F_c-specific antibodies

GαR F_c-specific antibodies (Abs) were diluted (to 0.5 or 1.0 mg/mL in PBS) and substituted for Protein A in the standard direct competitive ELISA protocol.

Competition step for ST analysis in milk and honey samples

Milk or diluted honey sample (75 µL) was added to the wells of the serum-treated microtiter plate, along with 25 µL of aqueous ST standards. To this was added 100 µL of HRP-ST diluted in PBST. All other procedures prior to and following the competition step were as outlined in the standard protocol.

ELISA data analysis

ELISA data were processed using *SOFTmax* and the following four-parameter equation for a sigmoidal curve (Fig. 2):

$$y = \frac{A - D}{1 + (x / C)^B} + D$$

where x = concentration of analyte (dose); y = response (i.e., absorbance); A = asymptote at low values of the x-axis; D = asymptote at high values of the x-axis; C = x-value corresponding to the midpoint between A and D values; B = rate of transition between A and D values.

RESULTS & DISCUSSION

AN INDIRECT COMPETITIVE ELISA for ST in honey had previously been developed in our laboratory (Sheth and Sporns, 1990). It was our intent to improve on the detection limits of that ELISA and to extend the methodology to permit analysis of ST in milk. There are no published reports of the details of immunological methods for analysis of ST in milk, and only a few commercial tests are available.

The majority of commercially available immunoassay kits for residue analysis are of the direct competitive variety. Direct competitive ELISAs are simple to perform, and require fewer reagents (thereby reducing cost) and less time for analysis than do indirect competitive counterparts. Competitive ELISAs are limited-reagent assays, that is they use limited amounts of antibody (Ab) (Kemeny and Chantler, 1988) and there is a competition between either immobilized or free hapten for a limited number of Ab binding sites. Soluble materials are removed, leaving behind only the immobilized antigen-antibody complexes (Roe, 1991).

Direct competitive ELISAs employ an enzyme-labeled immunoreactant. In labeled hapten direct assays, Ab is immobilized on the surface of a solid support (Fig. 1). A competition is established between free (unlabeled) antigen (Ag) in the sample and a known quantity of enzyme-labeled Ag for the limited number of Ag-binding sites on the immobilized Abs. Washing removes unreacted components, leaving behind immobilized Ag-Ab complexes, some of which will have been formed by the union of Ab with enzyme-labeled Ag. The intensity of the enzyme signal following incubation with its substrate is inversely proportional to the level of free Ag in the sample; quantitation is based on a standard curve (Fig. 2) (Clark and Engvall, 1980).

The primary or Ag-specific Ab in direct competitive ELISAs may be immobilized by simple adsorption to a plastic surface; however, the Ab may show a decreased Ag-binding capacity after coupling to a solid support (Ansari et al., 1978; Sankolli et al., 1987). The direct adsorption of Ab to the solid phase may also result in ineffective orientation of the binding sites. In particular, at low Ab concentration, the potential is high for the Abs to interact with the support, leaving fewer binding sites exposed for combining with Ag. It is most desirable to have "ends-on" immobilization in which Abs are immobilized

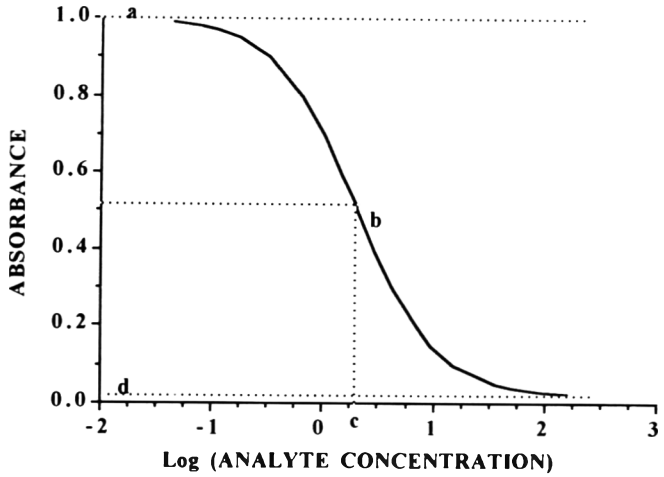

Fig. 2—Standard competitive inhibition curve for ELISAs.

Table 1—HRP-ST conjugates synthesized by glutaraldehyde cross-linking and periodate cleavage

Conjugate	mg ST	mg HRP	Molar ratio (ST:HRP)
G-Lite	3.0	11.3	46
G-Mod	5.7	10.8	91
G-Heavy	11.5	11.7	169
P-Lite	2.4	4.6	90
P-Mod	8.9	4.1	375
P-Heavy	81.5	4.6	3044

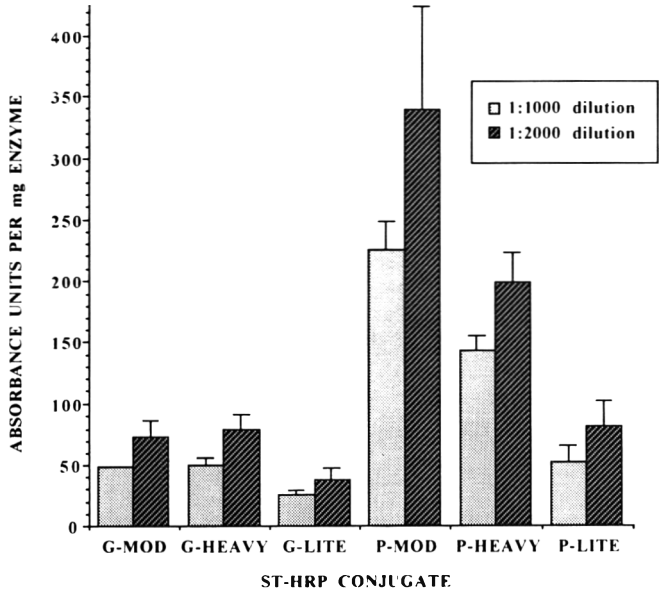

Fig. 3—Efficiency of HRP-ST conjugates. Points represent the mean value of two determinations; error bars indicate the standard deviation of the mean. Reaction time for color development was 10 min at room temperature; reaction was stopped by adding 50 µL/well 2 M sulfuric acid. Serum was diluted 1:25,000.

through portions distal to their binding sites, allowing full exposure of binding sites (Schramm and Paek, 1992). This may be enhanced by increasing the Ab density; however, higher levels of Ab can reduce the specificity due to steric hindrance of the Ag-binding site by surrounding Abs (Sankolli et al., 1987) and increase the detection limits of a competitive assay (Schramm and Paek, 1992). Precoating of plates with an intermediate protein or peptide that promotes proper Ab orientation can enable lower levels of Ab to be used, increasing assay sensitivity (Schneider and Hammock, 1992; Schramm and Paek, 1992).

In direct ELISAs employing an enzyme-labeled hapten, no amplification of enzyme signal occurs as is achieved in indirect ELISAs (Tijssen, 1985); therefore, the enzyme selected must have a high turnover number. Reagents are diluted out to decrease assay detection limits.

The format of the direct ELISA can greatly influence the sensitivity, maximum absorbance, and analysis time. If the proper strategy for incubation of the Ab, enzyme-analyte conjugate and free analyte is established, the free analyte can compete at very low levels. This would decrease the detection limit provided the affinity of the Abs is similar for both analyte and the enzyme-analyte tracer (Schneider and Hammock, 1992).

In order to evaluate the performance of the ELISAs, a detailed analysis of the ELISA data (i.e., the parameters of the sigmoidal curve; Fig. 2) was undertaken. In particular, a low C value was needed to indicate decreased detection levels. Low D values were important, indicating reduced background interference, while the magnitude of A-D must be considerable (i.e., about 0.2 O.D. units) to ensure accuracy of absorbance measurements (Skoog and West, 1976). Finally, a high correlation coefficient (r) was required when data were fitted to the sigmoidal curve.

Synthesis and characterization of ST-enzyme conjugates

Development of a direct competitive ELISA for ST required synthesis of a conjugate of ST and an enzyme marker. HRP is generally considered to be the enzyme of choice for ELISAs and was used as the marker enzyme. Our choice confirmed the results of Schneider and Hammock (1992) who showed that the sensitivity of a direct ELISA could be increased 100-fold when an HRP-hapten conjugate was used instead of an alkaline phosphatase-hapten conjugate.

Three HRP-ST conjugates were synthesized using the two-step glutaraldehyde method and designated G-Heavy, G-Mod, and G-Lite based on the assumed degree of ST substitution on the HRP molecule. Similarly, three HRP-ST conjugates were prepared using the periodate cleavage method and designated P-Heavy, P-Mod, and P-Lite (Table 1). We hypothesized that the ideal conjugate would consist of a ratio of hapten to enzyme of $\geq$ 1:1, as undersubstitution of the enzyme may lead to less presentation of the hapten to Ab; this may be influenced by the folding of the enzyme protein and steric considerations. In addition, under reaction conditions aimed at a low level of enzyme substitution with hapten, there may remain free, unconjugated enzyme which would not bind to Ab in the ELISA. Overloading of the enzyme with ST was to be avoided, as enzyme activity may be destroyed or the active site of the enzyme may be blocked at high substitution rates.

The six HRP-ST conjugates synthesized were evaluated on the basis of enzyme efficiency in a direct ELISA format (Fig. 3). Evidently HRP-ST conjugate P-Mod synthesized using a molar ratio of ST to HRP of 375:1 resulted in the most efficient use of enzyme, as shown by the high absorbance values/mg enzyme protein. HRP-ST conjugates produced using the periodate cleavage method were generally more effective than those produced using the glutaraldehyde cross-linking method (Fig. 3).

All conjugates showed greater efficiency at a higher rate of dilution (Fig. 3). Only a fixed amount of HRP-ST can bind, depending on the level of immobilized Ab. Thus, use of more concentrated solutions of HRP-ST would be wasteful, since unbound HRP-ST would be washed away prior to addition of enzyme substrate. Furthermore, in order to attain maximum sensitivity in ELISAs, immunoreactants should be present at the lowest concentrations possible. From these results, the moderately-loaded HRP-ST conjugate produced using the periodate cleavage method (P-Mod) was chosen for subsequent direct ELISA evaluations. The working dilution was set at 1:2,000.

Although the exact mechanism involved in the coupling of protein moieties by glutaraldehyde is not completely understood (Molin et al., 1978; Tijssen, 1985), the principal attachment sites

on HRP are the primary amino groups of amino acids, particularly lysine (Molin et al., 1978). HRP contains only six lysine residues (Welinder, 1979); however, in commercial preparations of HRP, the majority of the ε-amino groups of lysine are blocked by allylisothiocyanate or by the carbohydrates surrounding the molecule (Nakane and Kawaoi, 1974), making them unavailable for coupling. This may account for the similarity in efficiencies of the G-Mod and G-Heavy conjugates in our results. Probably in the moderately-loaded HRP-ST conjugate the maximum degree of substitution on the enzyme had been realized.

We believe this is the first report of the use of the periodate cleavage method for synthesis of HRP conjugates of sulfa drugs or of hapten molecules. Most published reports concern the use of the periodate cleavage method for conjugation of Abs or other large proteins, containing an abundance of ε-amino groups of lysine, to HRP. From our results, the method clearly can be employed to conjugate haptens with only weakly basic amino groups. For example, the pK_a for the ε-amino group of lysine is 10.53 (Whitaker, 1972), while the pK_a of the N^4-amino group of sulfathiazole is only 2.36 (Bell and Roblin, 1942). Moreover, the conjugates of ST and HRP prepared in this manner were more effective than those produced using the two-step glutaraldehyde method. Periodate cleavage is generally accepted as the best method for conjugation of HRP to macromolecules (Madersbacher et al., 1992, Tijssen, 1985) as it affords higher coupling efficiency than the two-step glutaraldehyde technique (Boorsma and Streefkerk, 1978). Our results are confirmatory to the currently held theory.

EMIT (enzyme-multiplied immunoassay technique) is a homogeneous immunoassay method which relies on a modulation of enzyme activity upon combination of the enzyme-labeled hapten with primary Ab (Jenkins, 1992). The use of an enzyme-labeled ST molecule in a direct competitive ELISA necessitated the testing of the conjugate for EMIT effects. If the enzyme signal had been moculated upon combination of the HRP-ST conjugate with varying amounts of Ab, it may have been possible to develop a simple homogeneous assay for ST. The highly-substituted HRP-ST conjugate (G-Heavy) was tested for EMIT effects (Fig. 4). Although a decrease in HRP activity occurred in the presence of serum as compared to that in the blank (without Ab), the activity of the enzyme remained relatively constant, regardless of serum level. Therefore, any changes in serum levels in developing the direct ELISA would not have influenced the amount of enzyme activity through interaction with the Ab.

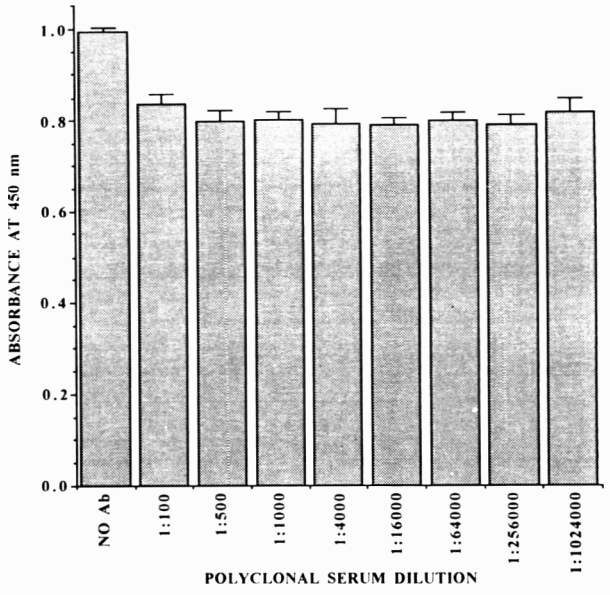

Fig. 4—Effect of serum dilution on activity of HRP-ST conjugate in solution. Points represent the mean absorbance value of six wells; error bars indicate the standard deviation of the mean. Reaction time for color development was 2 min at room temperature; reaction was stopped by adding 50 µL/well 2 M sulfuric acid. G-Heavy dilution was 1:1000.

Selection of direct ELISA format

Use of an enzyme-labeled hapten as the competitor conjugate necessitated the use of an Ab-coated solid phase. Key to the success of such ELISAs is the orientation of Abs on the solid phase, such that the binding sites are free to combine with hapten or tracer (Schramm and Paek, 1992).

Checkerboard ELISAs, performed using a variety of coating concentrations and Ab dilutions, indicated that the use of Protein A was more effective than GαR trapping Abs, which bind Abs in the portion of the molecule distal to the binding sites. It was also more effective than the simple adsorption of rabbit Abs to the microtiter plate in preparation for the competition step. Under identical conditions of serum and HRP-ST dilutions, higher absorbance values could be achieved on plates first treated with Protein A.

Competitive inhibition curves derived using Protein A-coated plates and those treated with GαR trapping Abs were similar. However, D values were higher using trapping Abs. Acceptable maximum absorbance values in the presence of extremely low amounts of ST (A values) for the Protein A precoating format were achieved in only 5 min. This indicated that a further reduction in serum levels was possible. Conversely, the use of trapping Abs necessitated longer incubation periods (20 min), even under conditions of higher levels of HRP-ST.

The simple adsorption of rabbit Abs to microtiter plates was unsatisfactory. Excessively high levels of serum (i.e., 1:1,600 dilution) were required for the development of appreciable color in the quantification step. Even after extended periods of incubation of substrate with bound HRP-ST (i.e., 30 min), absorbance values remained quite low as compared to those obtained using either of the precoating steps. This confirmed the findings of Schneider and Hammock (1992).

The use of either trapping Abs (GαR Abs specific for the F_c portion of the primary Ab) or Protein A as a preliminary step promoted a more favorable orientation of Abs on the microtiter plate by causing them to be bound through regions not involved in Ag binding (Schramm and Paek, 1992; Widjojoatmodjo et al., 1993). These Abs may also retain more of their native Ag binding ability. Schneider and Hammock (1992) also found the precoating step was advantageous to the performance of a direct competitive ELISA. They suggested the enhanced sensitivity and reproducibility may stem from the low concentrations of Ab and enzyme tracer that could be used under such conditions. Sankolli et al. (1987) similarly found that the use of trapping Abs was preferable to the simple coating of microtiter plates with primary Ab in promoting high binding capacity and low levels of well-to-well variation.

Schneider and Hammock (1992) did not report a significant difference between the use of Protein A or trapping Abs on the direct assay performance. However, Widjojoatmodjo et al. (1993) reported a distinct advantage to the use of Protein A as compared to goat anti-mouse Abs for the immobilization of murine monoclonal Abs on magnetic beads. They attributed the improved effectiveness of this format to the increased Ab-binding capacity of the Protein A-treated beads. The results of Widjojoatmodjo et al. (1993) also support our findings.

Direct competitive ELISA for sulfathiazole

The P-Mod conjugate was used to develop a direct competitive assay for aqueous solutions of ST (Fig. 5) The direct competitive ELISA for ST using aqueous standards operated optimally using a precoating step with Protein A, along with a serum

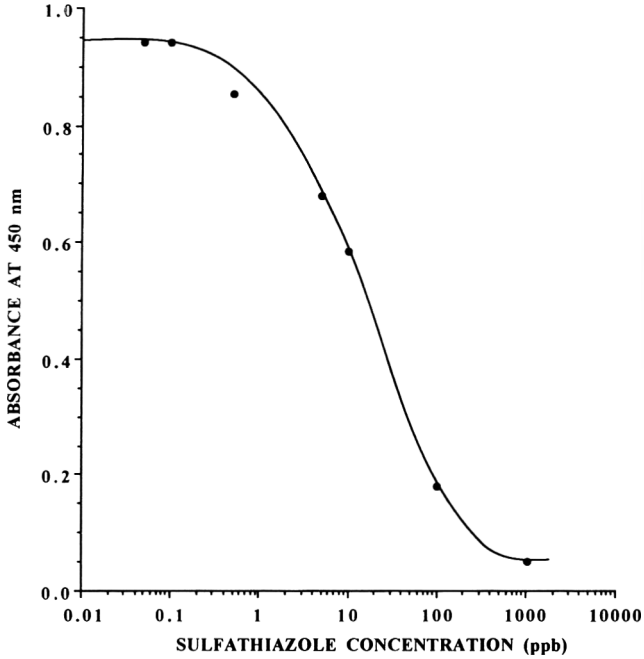

Fig. 5—Direct competitive ELISA standard curve for ST in aqueous solutions. Points represent the mean absorbance value of two wells; correlation coefficient (r) for the curve was 0.997. Reaction time for color development was 10 min at room temperature; reaction was stopped by adding 50 µL/well 2 M sulfuric acid. P-Mod dilution was 1:2000; serum was diluted 1:25,000.

Table 2—Direct ELISAs for sulfathiazole in milk[a]

Serum dilution	A value (A_{450})	B value	C value (ppb)	D value (A_{450})	r
1:25,000[b]	0.214	0.948	13.3	0.052	0.986
1:18,750	0.269	0.943	19.5	0.052	0.985
1:12,500	0.434	1.35	19.0	0.062	0.969
1:6,250	0.796	0.972	31.7	0.054	0.993

[a] Values represent the mean values calculated from the absorbance values of six wells; curve parameters were generated using *SOFTmax*. HRP-ST conjugate (P-Mod) dilution was 1:2000. Reaction of HRP with TMB and hydrogen peroxide was 30 min at room temperature; 50 µL/well 2 M sulfuric acid was added to stop the enzyme reaction.

[b] Curve for these data is presented in Fig. 6.

dilution of 1:25,000, and a 1:2000 dilution of P-Mod in the competition step.

Sulfathiazole analysis of milk using direct ELISA

Using the standard direct competitive protocol, an ELISA was developed for ST in undiluted milk samples (Table 2; Fig. 6). Using serum levels comparable to those used for the aqueous system (1:25,000), a longer incubation time for HRP with TMB-d was required (30 min as opposed to 10 min). This suggested that the milk interfered with the binding of the P-Mod conjugate to some degree. Increasing levels of serum increased the amounts of ST required to decrease maximum absorbance by 50% (C values) and reduced overall sensitivity of the test. A C value of 13.3 ppb ST was achieved using a serum dilution of 1:25,000 (Fig. 6). This represented an ST concentration in the original milk sample of 35.5 ppb after allowing for dilution during the competition step. Note that this value does not represent the detection limit of the test, but the concentration of ST required to reach the inflection point of the sigmoidal curve. If the detection limit of the assay was set at a 20% reduction in the A value, the detection limit for the assay would be 12.0 ppb ST in milk.

Sulfathiazole analysis of honey using direct ELISA

The first ELISA method for the analysis of ST in honey was an indirect ELISA which employed *o*-phenylenediamine as the detection reagent for HRP activity (Sheth and Sporns, 1990). The method required excessive amounts of serum for an indirect ELISA (1:32,000 dilution). While it eliminated the need for sample clean-up, it required a relatively high level of ST (300 ppb) to reduce maximum absorbance values by 50% (C values). Furthermore, very dilute honey samples (1:30 w/v) were required. Assil and Sporns (1991), who developed an indirect ELISA for the analysis of fumagillin in honey, indicated that more concentrated honey solutions were less likely to exhibit wide variability among samples.

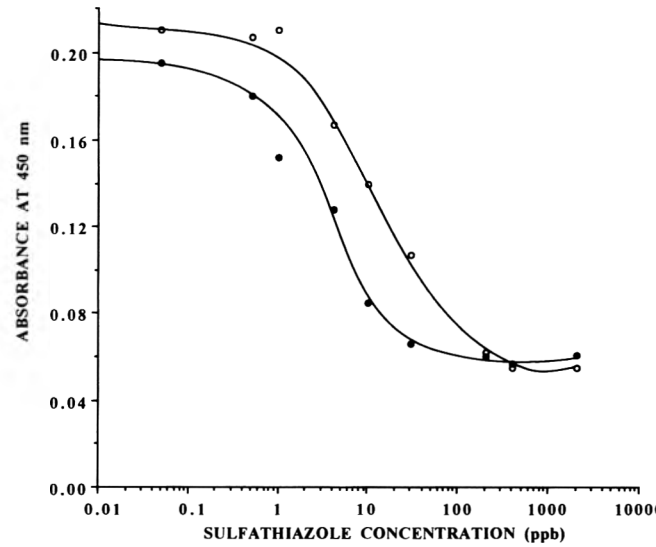

Fig. 6—Direct competitive ELISA standard curve for ST in milk and honey. Points represent the mean absorbance value of six wells; correlation coefficient (r) for the curve was 0.985 for milk and 0.966 for a 1:10 dilution of honey. Reaction time for color development was 30 min at room temperature; reaction was stopped by adding 50 µL/well 2 M sulfuric acid. P-Mod dilution was 1:2000; serum was diluted 1:18,750 for honey analysis (●) and 1:25,000 for milk analysis (○).

An objective of our study was to develop a direct competitive ELISA for ST in honey with greater sensitivity than reported by Sheth and Sporns (1990). We also needed an assay which would permit the analysis of minimally diluted honey samples. Minimal dilution (i.e., 1:2 or 1:5, w/v) with milli-Q water led to high background absorbances (D values) in the direct competitive ELISA for ST, resulting in ineffective competitive inhibition curves. By increasing the dilution rate of honey to 1:10 (w/v), the problem of high background absorbances was eliminated. As was observed in milk tests, reduction of serum levels led to increased sensitivity of the test for ST (Table 3). A C value of 3.3 ppb ST was achieved using a serum dilution of 1:18,750 (Fig. 6). This represented an ST concentration in the original honey sample of 88.0 ppb. Note that the data for honey were obtained using a 1:10 (w/v) dilution. The C value given above does not represent the detection limit of the test, but the concentration of ST required to reach the inflection point. If the detection limit of the assay was set at a 20% reduction in A value, the detection limit for the assay when used for ST analysis in honey would be 34.7 ppb ST.

At a low level of honey sample dilution, certain factors or substances in the honey samples were adversely affecting the assay. Since background absorbances were quite high, more concentrated honey samples apparently promoted a non-specific binding of the HRP-ST conjugate.

Since honey is typically a concentrated solution of glucose and fructose (White, 1975), we postulated that the high osmotic pressure of honey might affect the performance of the ELISA. However, when a honey analog was prepared and assayed at dilutions comparable to those causing unreliable results using honey samples, no interference was noted.

Table 3—Sulfathiazole detection in 1:10 (w/v) dilutions of honey[a]

Serum dilution	A value (A_{450})	B value	C value (ppb)	D value (A_{450})	r
1:25,000	0.12[illegible]	1.46	3.7	0.056	0.989
1:18,750[b]	0.194	1.07	3.3	0.058	0.966
1:12,500	0.32[illegible]	1.05	5.7	0.056	0.976
1:6,250	0.712	0.968	7.9	0.044	0.981

[a] Values represent the means calculated using the absorbance values of six wells; curve parameters were generated using *SOFTmax*. HRP-ST conjugate (P-Mod) dilution was 1:2,000. Reaction of HRP with TMB and hydrogen peroxide was 30 min at room temperature; 50 µL/well 2 M sulfuric acid was added to stop the enzyme reaction.

[b] Curve for these data is presented in Fig. 6.

Table 4—Effect of pH of a 1:3 (w/v) dilution of honey on direct ELISA parameters[a]

Honey pH	A value (A_{450})	B value	C value (ppb)	D value (A_{450})	r
3.7	0.471	0.799	23.6	0.259	0.975
4.0	0.389	1.14	21.7	0.174	0.954
5.0	0.713	0.723	27.5	0.161	0.977
6.3	0.615	1.04	25.7	0.079	0.983
7.1	0.704	0.991	14.3	0.064	0.978
8.1	0.694	1.02	11.3	0.049	0.985

[a] Values represent the means calculated using the absorbance values of 30 wells; curve parameters were generated using *SOFTmax*. Serum dilution was 1:6,250; HRP-ST conjugate (P-Mod) dilution was 1:2,000. Reaction of HRP with TMB and hydrogen peroxide was 30 min at room temperature; 50 µL/well 2 M sulfuric acid was added to stop the enzyme reaction.

We further postulated that minor constituents of honey samples, particularly hydrophobic substances such as wax and flavor volatiles, could interfere with the ELISA at low levels of dilution (where such components would not be diluted out). Concentrated honey samples (1:2 dilution, w/v) were pre-treated using C_{18} reverse-phase cartridges; however, high background absorbances remained a problem.

Finally, the effect of honey pH was investigated. By increasing the pH of a 1:3 (w/v) dilution of honey in water from the native value (~3.7) to 8, in ≈1 pH-unit increments, background absorbance interferences were greatly reduced (Table 4). The maximum absorbances (A values) of standard curves generally increased with increasing honey pH. The detection level of the assay also improved as honey pH increased, as indicated by the reduction in C values (Table 4).

Given the physiological conditions under which Abs are produced, we expected that a honey pH of ~7.1 would lead to the best results in a direct competitive ELISA for ST. The physiological pH of most mammals is slightly above neutrality (Pearson and Young, 1989); therefore, the immune response in which Ab production occurs in rabbits is in an approximately pH neutral environment. The pH can influence the ionization state of the Ag, which it does for ST (pK_a of 7.37 for N^1; Bell and Roblin, 1942), as well as the ionization states of individual amino acids that comprise the Ab protein. The charges on constituent amino acids may also influence the 3-dimensional structure of the Ab and therefore binding to Ag.

The problem of high background (D) values was effectively eliminated by adjusting the pH of concentrated honey samples to a more neutral value. However, little improvement was gained in terms of lowering C values from those using a 1:10 dilution of honey (Tables 2 and 3). Due to conversion of glucono-δ-lactone to gluconic acid salt during the pH adjustment, the procedure is time-consuming. We therefore decided that analysis for ST in honey would be most efficient using 1:10 dilutions of honey without pH adjustment.

Our direct ELISA for honey analysis improved upon that of Sheth and Sporns (1990) in two key ways. First, the C value of the direct system represented approximately a 3-fold lowering detection level. Second, using the direct ELISA, honey samples at their native pH needed only a 10-fold dilution to be analyzed, as compared to the previous 30-fold dilution. Note that the reduction in C values demonstrated in our results was a direct result of the protocol employed, since identical serum (i.e., from the same rabbit) was used in both studies.

CONCLUSIONS

DIRECT ELISAs WERE DEVELOPED for analysis of ST in milk and honey. Levels of ST required to decrease maximum absorbance values by 50% were 35.5 ppb for milk and 88.0 for honey. If the detection limits of such assays were set at a 20% reduction in maximum absorbance values, these values would be correspondingly reduced. We also demonstrated that ST could be conjugated to HRP more efficiently using periodate cleavage than using glutaraldehyde cross-linking. Periodate cleavage is particularly suitable for the conjugation of HRP due to the limited number of available lysine ε-amino groups. Our direct competitive ELISA method is the first rapid test which enables easy and rapid detection of ST at the 100 ppb level in honey. The detection limits of the ELISAs described here for both milk and honey might be further reduced with more effective polyclonal serum, which might be achieved by developing new serum using a minimally substituted injection conjugate.

REFERENCES

Abiân, J., Churchwell, M.I., and Korfmacher, W.A. 1993. High-performance liquid chromatography-thermospray mass spectrometry of ten sulfonamide antibiotics. Analysis in milk at the ppb level. J. Chromatogr. 629: 267–276.

Agarwal, V.K. 1992. High-performance liquid chromatographic methods for the determination of sulfonamides in tissue, milk and eggs. J. Chromatogr. 624: 411–423.

Anand, N. 1975. Sulfonamides and sulfones. In *Antibiotics III. Mechanisms of Action of Antimicrobial and Antitumor Agents*, J.W. Corcoran and F.E. Hahn (Ed.), p. 668–698. Springer-Verlag, New York.

Ansari, A.A., Bahuguna, L.M., and Malling, H.V. 1978. Comparison of double antibody and solid-phase immunoassays. J. Immunol. Methods 23: 219–226.

Argauer, R.J. 1986. Antibiotics in beekeeping. In *Agricultural Uses of Antibiotics*, W.A. Moats (Ed.), p. 35–48. American Chemical Society, Washington, DC.

Argauer, R.J., Shimanuki, H., and Knox, D.A. 1982. Determination of sulfathiazole in honey from medicated honey bee colonies by high-performance liquid chromatography on a cyano-amino polar phase. Environ. Entomol. 11: 820–823.

Assil, H.I. and Sporns, P. 1991. ELISA and HPLC methods for analysis of fumagillin and its decomposition products in honey. J. Agric. Food Chem. 39: 2206–2213.

Assil, H.I., Sheth, H., and Sporns, P. 1992. An ELISA for sulfonamide detection using affinity-purified polyclonal antibodies. Food Res. Int. 25: 343–353.

Barry, C.P. and MacEachern, G.M. 1983. Reverse-phase liquid chromatographic determination of sulfathiazole residues in honey. J. Assoc. Off. Anal. Chem. 66: 4–7.

Bell, P.H. and Roblin, R.O., Jr. 1942. Studies in chemotherapy. VII. A theory of the relation of structure to activity of sulfanilamide type compounds. J. Amer. Chem. Soc. 64: 2905–2917.

Belliardo, F. 1981. Determination of sulphonamide residues in honeys by high-pressure liquid chromatography. J. Apic. Res. 20: 44–48.

Boorsma, D.M. and Streekfkerk, J.G. 1978. Improved method for separation of peroxidase conjugates. In *Immunofluorescence and Related Staining Techniques*, W. Knapp, K. Holubar, and G. Wick (Ed.), p. 225–235. Elsevier/North Holland Biomedical Press, New York.

Brady, M.S. and Katz, S.E. 1988. Antibiotic/antimicrobial residues in milk. J. Food Prot. 51: 8–11.

Canadian Gazette. 1991. Food and drug regulations amendment No. 753. Part II. 125: 1479.

Clark, B.R. and Engvall, E. 1980. Enzyme-linked immunosorbent assay (ELISA): Theoretical and practical aspects. In *Enzyme-Immunoassay*, E.T. Maggio (Ed.), p. 167–179. CRC Press, Inc., Boca Raton, FL.

Diaz, T.G., Cabanillas, A.G., and Salinas, F. 1990. Rapid determination of sulfathiazole, oxytetracycline and tetracycline in honey by high-performance liquid chromatography. Anal. Lett. 23: 607–616.

Cribb, A.E., Miller, M., Leeder, J.S., Hill, J., and Spielberg, S.P. 1991. Reactions of the nitroso and hydroxylamine metabolites of sulfamethoxazole with reduced glutathione—Implications for idiosyncratic toxicity. Drug Metab. Dispos. 19: 900–906.

Dixon-Holland, D.E. and Katz, S.E. 1988. Competitive direct enzyme-linked immunosorbent assay for detection of sulfamethazine residues in swine urine and muscle tissue. J. Assoc. Off. Anal. Chem. 71: 1137–1140.

Dixon-Holland, D.E. and Katz, S.E. 1989. Direct competitive enzyme-linked immunosorbent assay for sulfamethazine residues in milk. J. Assoc. Off. Anal. Chem. 72: 447–450.

Dixon-Holland, D. and Katz, S.E. 1991. Competitive direct enzyme-linked immunosorbent screening assay for the detection of sulfamethazine contamination of animal feeds. J. Assoc. Off. Anal. Chem. 74: 784–789.

Eckert, J.E. 1947. Use of sulfa drugs in treatment of American foulbrood disease of honeybees. J. Econ. Entomol. 40: 41–44.

Fleeker, J.R. and Lovett, L.J. 1985. Enzyme immunoassay for screening sulfamethazine residues in swine blood. J. Assoc. Off. Anal. Chem. 68: 172–174.

Haseman, L. 1953. The sulfathiazole control of American foulbrood. Amer. Bee J. 93: 402–403.

Hays, V.W. 1986. Benefits and risks of antibiotics use in agriculture. In *Agricultural Uses of Antibiotics*, W.A. Moats (Ed.), p. 74–87. American Chemical Society, Washington, DC.
Hoffmeister, A., Suhren, G., and Heeschen, W. 1991. Enzyme immunoassays for screening sulfadimidine and sulfanilamide residues in milk. Milchwiss. 46: 712–717.
Horie, M., Saito, K., Nose, N., and Nakazawa, H. 1992. Simultaneous determination of sulfonamides in honey by liquid chromatography. J. AOAC Int. 75: 786–789.
Horwitz, W. 1981. Review of analytical methods for sulfonamides. Analytical methods for sulfonamides in foods and feeds. I. Review of methodology. J. Assoc. Off. Anal. Chem. 64: 104–130.
Huber, W.G. 1986. Allergenicity of antibacterial drug residues. In *Drug Residues in Animals*, A.G. Rico (Ed.), p. 33–50. Academic Press, Inc., New York
Jenkins, S.H. 1992. Homogeneous enzyme immunoassay. J. Immunol. Methods 150: 91–97.
Jürgens, U. 1982. Zur hochdruckflüssigchromatographischen Analyse von Arzneimittelrückständen in Honig. II. Chloramphenicol und Sulfathiazol. Z. Lebensm. Unters Forsch. 174: 208–210.
Kaneene, J.B. and Miller, R. 1992. Description and evaluation of the influence of veterinary presence on the use of antibiotics and sulfonamides in dairy herds. J. Amer. Vet. Med. Assoc. 201: 68–76.
Katz, S.E. 1986. Microbiological assay procedures for antibiotic residues. In *Agricultural Uses of Antibiotics*, W.A. Moats (Ed.), p. 142–153. American Chemical Society, Washington, DC.
Kemeny, D.M. and Chantler, S. 1988. An introduction to ELISA. In *ELISA and Other Solid Phase Immunoassays. Theoretical and Practical Aspects*, D.M. Kemeny and S.J. Challacombe (Ed.), p. 1–29. John Wiley & Sons, New York.
Larocque, L., Carignan, G., and Sved, S. 1990. Sulfamethazine (sulfadimidine) residues in Canadian consumer milk. J. Assoc. Off. Anal. Chem. 73: 365–367.
Long, A.R., Short, C.R., and Barker, S.A. 1990. Method for the isolation and liquid chromatographic determination of eight sulfonamides in milk. J. Chromatog. 502: 87–94.
McCaughey, W.J., Elliott, C.T., and Crooks, S.R.H. 1990. Determination of sulphadimidine in animal feedstuffs by an enzyme-linked immunoassay. Food Addit. Contam. 7: 259–264.
Madersbacher, S., Wolf, H., Gerth, R., and Berger, P. 1992. Increased ELISA sensitivity using a modified method for conjugating horseradish peroxidase to monoclonal antibodies. J. Immunol. Methods 152: 9–13.
Molin, S.-O., Nygren, H., Dolonius, L., and Hansson, H.-A. 1978. A kinetic study of the reaction between glutaraldehyde and horseradish peroxidase. J. Histochem. Cytochem. 26: 1053–1056.
Nakane, P.K. and Kawaoi, A. 1974. Peroxidase-labeled antibodies: Preparation and application for the localization of antigens. J. Histochem. Cytochem. 14: 929–931.
Neidert, E., Baraniak, Z., and Sauvé, A. 1986. Rapid quantitative thin-layer chromatographic screening procedure for sulfathiazole residues in honey. J. Assoc. Off. Anal. Chem. 69: 641–643.
Parks, O.W. 1982. Screening test for sulfamethazine and sulfathiazole in swine liver. J. Assoc. Off. Anal. Chem. 65: 632.
Pearson, A.M. and Young, R.B. 1989. *Muscle and Meat Biochemistry*, p. 398, Academic Press, Inc., New York.
Roe, R.M. 1991. Enzyme-linked immunosorbent assay of small-molecular-weight toxicants. In *Pesticides and the Future: Toxicological Studies of Risks and Benefits*, E. Hodgson, R.M. Roe, and N. Motoyama (Ed.), p. 273–287. N. Carolina State Univ., Raleigh, NC.
Roudaut, B. and Moretain, J.P. 1990. Sulphonamide residues in milk of dairy cows following intravenous injection. Food Addit. Contam. 7: 527–533.
Sankolli, G.M., Stott, R.A., and Kricka, L.J. 1987. Improvement in the antibody binding characteristics of microtiter wells by pretreatment with anti-IgG F_c immunoglobulin. J. Immunol. Methods 104: 191–194.
Schiffmann, A.P., Schütz, M., and Wiesner, H.U. 1992. False negative and positive results in testing for inhibitory substances in milk. I. The influence of antibiotic residues in bulk milk on lactic acid production of starter cultures. Milchwiss. 47: 712–715.
Schneider, P. and Hammock, B.D. 1992. Influence of the ELISA format and the hapten-enzyme conjugate on the sensitivity of an immunoassay for s-triazine herbicides using monoclonal antibodies. J. Agric. Food Chem. 40: 525–530.
Schramm, W. and Paek, S.-H. 1992. Antibody-antigen complex formation with immobilized immunoglobulins. Anal. Biochem. 205: 47–56.
Schwartz, D.P. and Sherma, J. 1986. Practical screening procedure for sulfathiazole in honey. J. Assoc. Off. Anal. Chem. 69: 72–74.
Sheth, H.B. and Sporns, P. 1990. Enzyme immunoassay for screening of sulfathiazole in honey. J. Assoc. Off. Anal. Chem. 73: 871–874.
Sheth, H.B. and Sporns, P. 1991. Development of a single ELISA for detection of sulfonamides. J. Agric. Food Chem. 39: 1696–1700.
Sheth, H.B., Yaylayar, V.A., Low, N.H., Stiles, M.E., and Sporns, P. 1990. Reaction of reducing sugars with sulfathiazole and importance of this reaction to sulfonamide residue analysis using chromatographic, colorimetric, microbiological, or ELISA methods. J. Agric. Food Chem. 38: 1125–1130.
Singh, P., Ram, B.P., and Sharkov, N. 1989. Enzyme immunoassay for screening of sulfamethazine in swine. J. Agric. Food Chem. 37: 109.
Skoog, D.A. and West, D.M. 1976. *Fundamentals of Analytical Chemistry*, 3rd ed., p. 549. Holt, Rinehart and Winston, Toronto.
Stecyk, T. 1987. "Drug-free" honey hard on Canadian beekeepers. Amer. Bee J. (Nov.): 761, 802.
Takeda, N. and Akiyama, Y. 1991. Pre-column derivatization of sulfa drugs with fluorescamine and high-performance liquid chromatographic determination at their residual levels in meat and meat products. J. Chromatogr. 558: 175–180.
Takeda, N. and Akiyama, Y. 1992. Rapid determination of sulfonamides in milk using liquid chromatographic separation and fluorescamine derivatization. J. Chromatogr. 607: 31–35.
Tijssen, P. 1985. *Practice and Theory of Enzyme Immunoassays*. Elsevier Science Publishing Company, Inc., New York.
Van Poucke, L.S.G., Depourcq, G.C.I., and Vanpeteghem, C.H. 1991. A quantitative method for the detection of sulfonamide residues in meat and milk samples with a high-performance thin-layer chromatographic method. J. Chromatogr. Sci. 29: 423–427.
Weber, J.D. and Smedley, M.D. 1983. Liquid chromatographic determination of sulfamethazine in milk. J. Assoc. Off. Anal. Chem. 72: 445–446.
Welinder, K.G. 1979. Amino acid sequence studies of horseradish peroxidase. Eur. J. Biochem. 96: 483–502.
Whitaker, J.R. 1972. *Principles of Enzymology for the Food Sciences*. Marcel Dekker, Inc., New York.
White, J.W. Jr. 1975. Composition of honey. In *Honey*, E. Crane (Ed.). William Heinemann Ltd., Toronto.
Widjojoatmodjo, M.N., Fluit, A.C., Torensma, R., and Verhoef, J. 1993. Comparison of immunomagnetic beads coated with protein A, protein G, or goat anti-mouse immunoglobulins. Applications in enzyme immunoassays and immuno-magnetic separation. J. Immunol. Methods 165: 11–19.
Wilson, M.B. and Nakane, P.K. 1978. Recent developments in the periodate method of conjugating horseradish peroxidase (HRPO) to antibodies. In *Immunofluorescence and Related Staining Techniques*, W. Knapp, K. Holubar, and G. Wick (Ed.), p. 215–224. Elsevier/North Holland Biomedical Press, New York.
Woodward, K.N. 1991. The Regulation of carcinogenic drugs: The carcinogenicity of sulphadimidine—Risk assessment for veterinary use in food-producing animals. Human Exp. Toxicol. 10: 84.
Woodward, K.N. 1992. Carcinogenicity of sulphadimidine. Human Exp. Toxicol. 11: 60–61.
Zomer, E., Saul, S., and Charm, S.E. 1992. HPLC receptogram: A method for confirmation and identification of antimicrobial drugs by using liquid chromatography with microbial receptor assay. I. Sulfonamides in milk. J. AOAC Int. 75: 987–993.
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ε-(γ-Glutamyl)lysine Crosslink Distribution in Foods as Determined by Improved Method

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ABSTRACT

Quantitative analysis of ε-(γ-glutamyl)lysine crosslink in 127 foods was achieved with a preliminary separation by reverse phase-HPLC before *o*-phthalaldehyde derivatization to remove interfering peaks. ε-(γ-Glutamyl)lysine was detected in 96 foods and its contents ranged from 0.2 to 135 μmol/100g protein. High levels were found in fish paste products, processed fish, shellfish, meats and soybeans, and raw poultry organs. For fish and meats, the level of ε-(γ-glutamyl)lysine in processed foods and fish paste was relatively higher than that in raw materials. The improved procedure could be applied for screening materials with transglutaminase activities.

Key Words: fish, seafood, meat, peptides, glutamyl-lysine crosslinks

INTRODUCTION

FOOD TEXTURE is an important property of foods, but its basis is complex, because it is affected by many factors. Studies have been conducted to investigate such factors as physicochemical and rheological properties of foods (Bourne, 1982; Rao and Steffe, 1992). Texture is closely related to human responses, i.e., mastication period, secretion of saliva and chewing energy (Kowata et al., 1987; Sakamoto et al., 1989).

As related to food proteins, many kinds of bonds or crosslinks are important for texture development. Such bonds and crosslinks might result in important changes in textural properties, as well as chemical and nutritional properties. Hydrogen bonds, electrostatic and hydrophobic interactions and disulfide bonds generally are responsible for construction and maintenance of protein structure. In complicated systems such as meat, however, a variety of crosslinks, including ε-(γ-glutamyl)lysine [ε-(γ-Glu)Lys] crosslinks and crosslinks through aldol condensation, are believed to be closely related to texture. Such bonds are responsible for formation of strong but elastic tissues in living organisms (Singh, 1991). Whitaker (1977) suggested that the possibility of such crosslinks in proteins might be used to fabricate texturized products.

Many attempts to improve textural properties of foods through formation of ε-(γ-Glu)Lys crosslinks by the catalytic action of transglutaminases (TGase) have been reported (Ikura et al., 1980a, 1980b; Motoki and Nio, 1983; Kurth and Rogers, 1984; Kato et al., 1991). These enzymes have been found in many cells, organisms and tissues. The crosslink results from interaction between the γ-carboxyamide group of peptide-bound glutamine residues and ε-amino group of peptide-bound lysine residues (Pisano et al., 1968; Williams-Ashaman et al., 1972; Folk and Chung, 1973; Folk and Finlayson, 1977; Birckbichler et al., 1981). Intermolecular crosslinks in several proteins were induced using guinea pig liver TGase and bovine plasma TGase (Ikura et al., 1980a, 1980b; Motoki and Nio, 1983; Kurth and Rogers, 1984; Bercovici et al., 1987; Kato et al., 1991).

Motoki and Nio (1983) and Nio et al. (1985, 1986a, 1986b) purified the TGase from guinea pig livers and reported that many other food proteins, as well as soybean protein and bovine casein, were substrates and formed gels on incubation with the enzyme. Such gels have unique characteristics; gelation may occur without thermal treatment; highly elastic gels are formed; heat- and water-resistant films can be made, and lipids and lipid-soluble materials can be encapsulated. Upon the discovery of an extracellular microbial TGase produced by a variant of *Streptoverticillium mobaraense* (Ando et al., 1989; Washizu et al., 1994), utilization of TGase in food industries was developed (Nonaka et al., 1989, 1992, 1994; Tanaka et al., 1990). Besides enzymes of microbial origin, endogenous TGases were found in fish flesh. These reportedly formed the ε-(γ-Glu)Lys crosslink in fish proteins and changed textural properties of fish sol during processing (Seki et al., 1990; Tsukamasa and Shimizu, 1990, 1991; Kimura et al., 1991; Sato et al., 1992; Kumazawa et al., 1993). TGase activity also has been reported in other foods, such as pea, alfalfa, beet (Icekson et al., 1987; Margosiak et al., 1990; Signorini et al., 1991).

We hypothesized that TGases were widely distributed in food materials and thus, could influence protein structures and/or textures. If specific foods with high activity were found, they would possibly be good sources of TGase. However, detection of TGase activity in foods is not simple because the TGase must be purified from other components, which could interfere with the activity assay.

The analysis for ε-(γ-Glu)Lys crosslink, the catalytic product of TGases, would enable detection and screening of endogenous TGases in many foods. Sensitive quantification of the ε-(γ-Glu)Lys crosslink has been reported with exhaustive proteolytic digestion and subsequent derivatization with *o*-phthalaldehyde (OPA). High performance liquid chromatography (HPLC) separation was then used to resolve the peptide (Griffin et al., 1982, 1984; Beninati et al., 1988). Complete resolution of the ε-(γ-Glu)Lys derivatives was affected by other peaks and the appearance of large unknown peaks (Griffin et al., 1982; Nonaka et al., 1989; Tanaka et al., 1990; Tarcsa and Fesus, 1990; Kimura et al., 1991). Precise quantitative analysis has been difficult.

Our objectives were to improve the OPA derivatization method for investigation of the distribution of the ε-(γ-Glu)Lys crosslink in several foods and determine which types contained high levels of the crosslink.

MATERIALS & METHODS

Reagents

Synthetic ε-(γ-Glu)Lys was purchased from Sigma Chem. Co. (St. Louis, MO). Amino acid standard mixtures (Type H: a mixture of 17 amino acids and ammonium chloride), trifluoroacetic acid (TFA: protein sequencing grade), acetonitrile (HPLC grade), tetrahydrofuran (THF), potassium acetate (protein sequencing grade) and o-phthalaldehyde (OPA) were purchased from Wako Pure Chem. Co., Osaka, Japan. All other reagents were analytical grade.

Pronase from *Streptomyces griseus* and carboxypeptidase A from bovine pancreas were purchased from Boehringer Mannheim GmbH (Germany). Leucine aminopeptidase and prolidase from porcine kidney were purchased from Sigma Chem. Co..

High-performance liquid chromatography (HPLC) apparatus

The HPLC apparatus consisted of a Waters Model 600 multisolvent system; a Model 490 programmable multiwavelength detector, a Model

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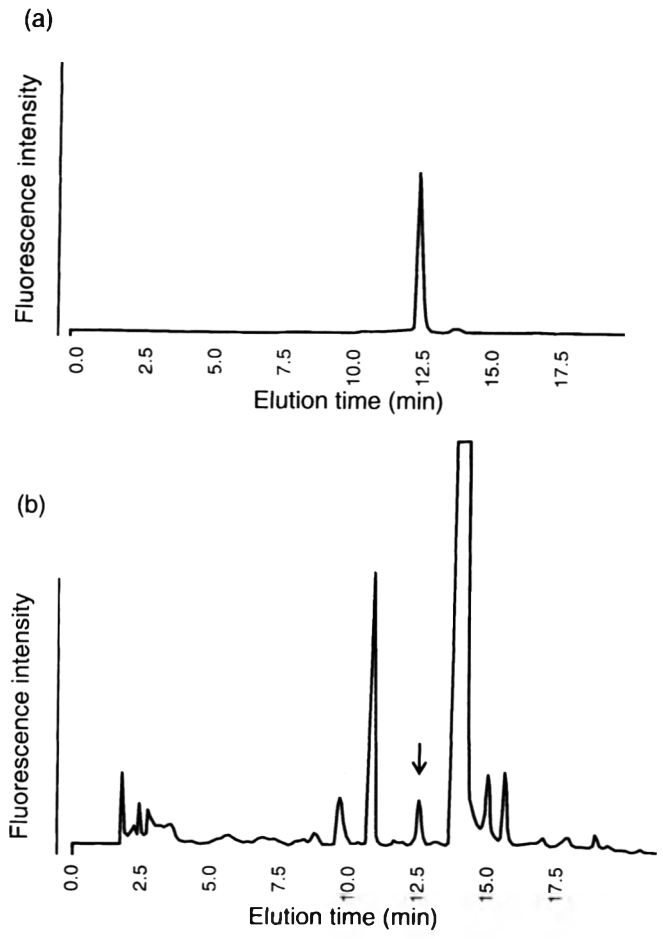

Fig. 1—Chromatogram of ε-(γ-glutamyl)lysine dipeptide. (a) Standard synthetic ε-(γ-glutamyl)lysine (100 picomoles). (b) Natural ε-(γ-glutamyl)lysine from proteolytic digest of chicken heart. The arrow shows the peak of the dipeptide.

420 scanning fluorescence detector; and a Model 741 data module (ATT256), all from Waters Division Japan Millipore Ltd., Co., Tokyo, Japan).

Preparation of protein samples

Sample foods (127, of several types) obtained from local markets consisted of 34 fish and shellfish, 27 meats, 8 dairy products, 17 beans and seeds, 11 cereals, 9 confectioneries and snacks, 7 seasonings and 14 vegetables and fruits. These included raw and processed products. Food samples (50g each) were homogenized with 100 mL distilled water. The homogenate was centrifuged at 3000 g for 10 min at 4°C. Solid fat was separated and removed from the system. Dried weight (%) was determined as a ratio of lyophilized weight to wet weight of the 50 g sample. Protein concentration of samples was measured by the Kjeldahl method using a Kjeltec Model 1030 auto analyzer and a Model 1026 distilling unit (AOAC, 1990).

Proteolytic digestion

To obtain the ε-(γ-Glu)Lys dipeptide, exhaustive proteolytic digestion was carried out according to the method of Griffin et al. (1982). A 3 mL aliquot of 0.1M borate buffer (pH 8.0) and a crystal of thymol were added to the protein sample (20 mg protein). Proteolytic digestion of this mixture was carried out by sequential addition of proteolytic enzymes (Nonaka et al., 1989). Pronase was applied in two additions of 0.4 units/mg protein, each for a duration of 24 hr at 37°C. After inactivation of pronase by heating at 100°C for 10 min, digestion was continued at 37°C for 24 hr by adding leucine aminopeptidase (0.4 units/mg protein) and prolidase (0.45 units/mg protein). After 24 hr leucine aminopeptidase was again added for the same duration of time. Finally, carboxypeptidase A (0.2 units/mg protein) was added, and the mixture was digested for another 24 hr at 37°C. Ratio of enzyme to sample protein was about 0.003 for each enzyme and 0.018 for the total enzymes. After heat inactivation (100°C for 10 min), the proteolytic digest was lyophilized.

Preliminary chromatographic fractionation

A preliminary fractionation was accomplished by filtration on an Inertsil ODS-2 column (150 mm × 6.0 mm i.d., GL Science, Tokyo, Japan) at 1 mL/min, with the column at 2°C. Elution was with 0.1% TFA aqueous solution, and the column was washed with acetonitrile containing 0.1% TFA. Detection was at 210 nm with a sensitivity of 1.0 AUFS. Synthetic ε-(γ-Glu)Lys (1 mM) was used as the standard and eluted at 12.5 min.

The dried digests (unknowns) were subjected to the same fractionation procedure as for the standard. Dried digests were dissolved in 7.5 mL distilled water, and the solution was filtered through a 0.45 μm filter (Columnguard, Millipore, Bedford, MA). An aliquot (100 μL) of this filtrate without the standard ε-(γ-Glu)Lys was applied to the column and each sample was fractionated for 2 min, from 45 sec prior until 1 min 15 sec after the retention time of the standard dipeptide (12.5 min). The fractionated samples were lyophilized.

HPLC separation of ε-(γ-Glu)Lys

Precolumn derivatization with OPA was according to the method of Griffin et al. (1982). A dried fractionated sample was mixed with 500 μL OPA reagent and kept for 3 min at room temperature (25°C). An aliquot of 100 μL from this reaction mixture was applied on a reverse phase-HPLC with a Zorbax BP-C_8 column (250 × 4.6 mm i.d., GL Science, Tokyo, Japan).

Elution was with binary linear multistep solvent gradients, in which Solution A consisted of 20 mM potassium acetate (pH 5.5) with 1% (v/v) THF and Solution B, methanol with 1% (v/v) THF. Separation was accomplished with a linear gradient of 20% to 95% methanol at 2 mL/min for 20 min and the column at 40°C. For fluorescence detection, excitation was set at 334 nm and emission at 440 nm and the gain was set at 8. ε-(γ-Glu)Lys dipeptide was used as the standard and retention time for ε-(γ-Glu)Lys contained in the sample was estimated by using the standard ε-(γ-Glu)Lys.

To confirm ε-(γ-Glu)Lys obtained with OPA analysis, an ion-exchange HPLC (amino acid analyzer Beckman system 6300E) was used. After preliminary chromatographic purification, the sample was separated and concentrated by using thin-layer chromatography. This was performed on Spotfilm® (microcrystalline cellulose, Tokyo Kasei Kogyo Co., Tokyo, Japan) and the spots were detected with ninhydrin. Solvent for the separation consisted of n-butanol-pyridine-acetic acid-water (15 : 10 : 3 : 12) (Kirchner, 1978). Following separation and concentration ion exchange HPLC was applied.

Analysis of variance (ANOVA)

To determine variance among food treatments (raw, cooked, fish paste products, processing), a one-way analysis-of-variance was performed (Ichihara, 1990; Ishihara et al., 1990).

RESULTS & DISCUSSION

HPLC ELUTION PROFILES of the standard synthetic ε-(γ-Glu)Lys (100 picomoles) and natural ε-(γ-Glu)Lys in chicken heart eluted at the same retention time (12.5 min, Fig. 1). Nonaka et al. (1989) reported that any amino acids in the standard mixture did not coelute with the standard ε-(γ-Glu)Lys and that was confirmed in our results (data not shown). Thus, excellent resolution was achieved after preliminary fractionation. The relationship between peak area and quantity of synthetic ε-(γ-Glu)Lys was linear ($r = 0.999$) (Fig. 2). All analyses were performed within the linearly correlated range. Of the standard ε-(γ-Glu)Lys, 100% was collected in the preliminary fractionation. Molar concentrations of glutamate and lysine in 6N HCl hydrolyzates of the samples equalled that of the fractionated ε-(γ-Glu)Lys (data not shown). Deviations were about 4–12% for OPA analysis in triplicate.

In the method of Griffin et al. (1982), the ε-(γ-Glu)Lys derivative was affected by other amino acid peaks and large, unknown peaks. The method was modified so that interfering

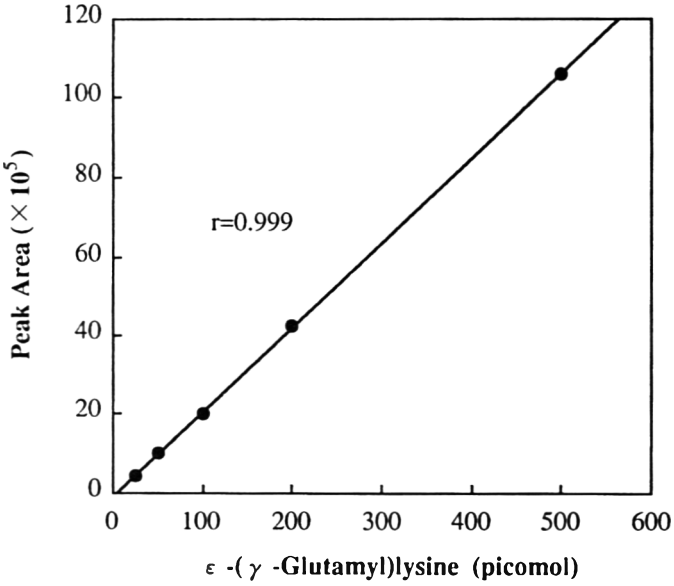

Fig. 2—Relationship between HPLC peak area and amount of ε-(γ-glutamyl)lysine.

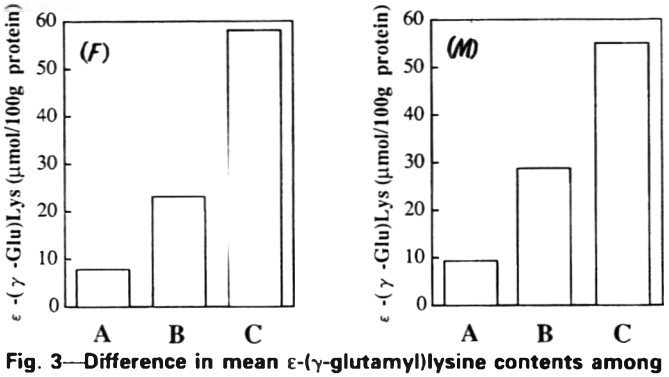

Fig. 3—Difference in mean ε-(γ-glutamyl)lysine contents among raw and processed foods. (F) Fish and shellfish: (A) raw fish and shellfish (14 kinds); (B) cooked fish products (11 kinds); (C) fish paste products (9 kinds). (M) Meats: (A) raw meats (5 kinds); (B) cooked meat products (10 kinds); (C) raw organs (12 kinds).

peaks were removed by a reverse phase-HPLC before derivatization with OPA. The fraction containing ε-(γ-Glu)Lys could be condensed and the sensitivity improved. Blank sample treated similarly showed no interfering peaks at the ε-(γ-Glu)Lys retention time. Fluorescence intensity of the OPA derivatives depends on the time lapse after derivatization (Griffin et al., 1982). Based upon the preliminary fractionation and precise control of derivatization time and temperature, quantitative analysis of ε-(γ-Glu)Lys was achieved.

ε-(γ-Glutamyl)lysine was detected in 96 foods and ranged from 0.2 to 135 μmol/100g protein. Results indicated wide occurrence and distribution of ε-(γ-Glu)Lys in foods. Large variations in the ε-(γ-Glu)Lys content were observed in fish, shellfish and meats (mean ±SD, 31.3 ± 31.0 μ mol/100 g protein), as compared with other food groups (mean ±SD, 7.2 ± 10.6 μmol/100 g protein). However, many foods showed high levels of ε-(γ-Glu)Lys.

In fish paste products, *Kamaboko* showed high ε-(γ-Glu)Lys levels [seven *Kamabokos* (mean ±SD) 55.2 ± 34.7 μmol/100 g protein] and imitation crab meat had the highest content (101.6 μmol/100g protein). Additionally, high levels were found in canned fish (sardine—33.9 μmol/100 g protein; tuna—11.2 μmol/100g protein) and raw fish (crab meat—34.3 μmol/100 g protein). In meats, high levels were found in raw organs (five cow organs—71.9 ± 49.8 μmol/100g protein; five chicken organs—46.2 ± 37.5 μmol/100g protein; two pork organs—13.6 and 55.9 μmol/100g protein). Cow colons had the highest content (135.4 μmol/100g protein). High levels were also found in canned processed meat (two kinds of stewed beef, corned beef, and sliced ham—39.4 ± 21.5 μmol/100 g protein). The variance of ε-(γ-Glu)Lys within a given food group probably was due to differences in composition of products or processing conditions.

In beans and peas, the highest level was found in *Kinako* (roasted soybean flour—50.3 μmol/100 g protein). ε-(γ-Glu)Lys was also found in soy bean curd products (six types of products—10.7 ± 12.4 μmol/100g protein). In most dairy products, such as cow's milk, yogurt and four kinds of cheese, ε-(γ-Glu)Lys was not found. However, it was found in cooked meltable cheese (16.7 μmol/100g protein).

ε-(γ-Glu)Lys was in very low concentrations in confectioneries and snacks; however, corn puffs (31 μmol/100g protein) and rice crackers (24.6 μmol/100 g protein) showed high levels. It was also found in seasonings (beef extract), in vegetables (*hoshi-shiitake*; dried mushroom), and in cereals (corn flakes—18.4 μmol/100g protein; white bread—11.1 μmol/100g protein; retorted rice—11.1 μmol/100g protein).

Tsukamasa et al. (1993) reported that ε-(γ-Glu)Lys crosslink formation occurred in sardine myofibrils during setting and Kumazawa et al. (1993) reported that formation of ε-(γ-Glu)Lys crosslink occurred through transglutaminase reaction in cured horse mackerel. High ε-(γ-Glu)Lys content in commercial fish paste products was demonstrated by our experiments.

The existence of TGases in mammals and chicken and the occurrence of ε-(γ-Glu)Lys in animal organs, tissues and body fluids have been reported (Folk and Finlayson, 1977). Although animals such as rats and guinea pigs are generally not consumed as human foods, they were tested. Only chicken heart myofibrils or chicken breast muscle ε-(γ-Glu)Lys has been reported (Folk and Finlayson, 1977; Loewy and Matacic, 1981). In our study, high ε-(γ-Glu)Lys levels were found in many raw organ foods (poultry organs are common in Japan, China, Korea and Europe). These results suggested that ε-(γ-Glu)Lys could be formed by catalytic action of TGases.

The mean ε-(γ-Glu)Lys content of cooked meat products, such as canned stewed beef, canned sliced ham, canned corn beef, grilled pork, deep-fried chicken and stewed pork was higher than that of raw meats. These results indicate that ε-(γ-Glu)Lys could also be generated during heat treatment, since it is formed as a consequence of thermal treatment of protein (Otterburn, 1983). ε-(γ-Glu)Lys, found in keratin (Asquith and Otterburn, 1971), milk proteins (Schmitz et al., 1976), muscle proteins (Hurrell et al., 1976), and other proteins (Otterburn et al., 1977), was formed during heating and increased with severity of heating. In heated proteins, it was assumed to be generated from a condensation reaction between the free γ-carboxyl of glutamic acid and ε-amino groups of lysine or formed through an aminolysis of the γ-carboxylamide of glutamine with the ε-amino group of lysine. These reactions are independent of transglutaminase activity (Asquith et al., 1974). In such cases, the heat treatment was severe (4–27 hr at 121°C). As for other foods, the influence of heat treatment on ε-(γ-Glu)Lys content has not been reported. Generally most proteins as well as endogenous TGases in food sources are heat labile, inactivated during cooking or canning.

Differences in ε-(γ-Glu)Lys between raw and processed foods (fish and shellfish, cooked fish products and fish paste products) were found (Fig. 3F). Fish paste products were imitation crab meat and several kinds of *kamaboko*, and cooked fish products were several kinds of canned or cooked fish. Significant differences were observed among each group ($P < 0.01$). The mean ε-(γ-Glu)Lys content of fish paste products was the highest and that of cooked fish products second. These values were higher than those of raw samples. ε-(γ-Glu)Lys in fish paste products are hypothesized to be generated during processing by the catalytic action of endogenous TGases. ε-(γ-Glu)Lys in cooked fish

products are also probably formed by the TGase. However, ε-(γ-Glu)Lys was also thought to be generated by heat treatment during processing.

Mean concentrations of ε-(γ-Glu)Lys in raw meats, cooked meat products and raw organs were compared (Fig. 3e). Cooked meat products showed higher concentrations than raw meats. Significant differences were observed among each group ($P < 0.05$). Raw organs were cow, pork and chicken livers and cooked meat products were canned stewed beef, grilled pork, deep-fried chicken and stewed pork. Raw meats were cow flank and round, pork flank and inside ham and chicken thigh. The ε-(γ-Glu)Lys content was highest in raw organs and that of cooked meat products was second. ε-(γ-Glu)Lys in raw organs is hypothesized to be formed by the catalytic action of endogenous TGases (Folk and Finlayson, 1977). ε-(γ-Glu)Lys in cooked meats products is also probably generated by catalytic action of TGase. However, heat treatment was also capable of forming the dipeptide. On heat treatment, TGase would be inactivated by heat denaturation, whereas ε-(γ-Glu)Lys formation without TGase could occur (Asquith et al., 1974).

We did not determine whether ε-(γ-Glu)Lys found in cooked foods products was formed through catalytic action of TGase or by heat treatment. However, our results indicate that our improved analysis for ε-(γ-Glu)Lys could be used to screen materials containing TGases. Those foods with high ε-(γ-Glu)Lys levels, particularly those not subjected to heat treatment, may show high TGase activity (Seki et al., 1990; Folk and Finlayson, 1977).

REFERENCES

Ando, H., Adachi, M., Umeda, K., Matsuura, A., Nonaka, M., Uchio, R., Tanaka, H., and Motoki, M. 1989. Purification and characteristics of a novel transglutaminase derived from microorganisms. Agric. Biol. Chem. 53: 2613–2617.

AOAC. 1990. *Official Methods of Analysis*, 15th ed. Association of Official Analytical Chemistry, Washington, DC.

Asquith, R.S. and Otterburn, M.S. 1971. Self-crosslinking in keratin under the influence of dry heat. Appl. Polym. Symp. 18: 277–287.

Asquith, R.S., Otterburn, M.S., and Sinclair, W.J. 1974. Isopeptide crosslinks—their occurrence and importance in protein structure. Angew. Chem. Int. 13: 514–520.

Beninati, S., Martinet, N., and Folk, J.E. 1988. High-performance liquid chromatographic method for the determination of ε-(γ-glutamyl)lysine and mono- and bis-γ-glutamyl derivatives of putrescine and spermidine. J. Chromatogr. 443: 329–335.

Bercovici, D., Gaertner, H.F., and Puigserver, A.J. 1987. Transglutaminase-catalyzed incorporation of lysine oligomers into casein. J. Agric. Food Chem. 35: 301–304.

Birckbichler, P.J., Orr, G.R., Patterson, M.K., Jr., Conway, E., and Carter, H.A. 1981. Increase in proliferative markers after inhibition of transglutaminase. Proc. Natl. Acad. Sci. 78: 5005–5008.

Bourne, M.C. 1982. *Food Texture and Viscosity*. Academic Press, New York.

Folk, J.E. and Chung, S.I. 1973. Molecular and catalytic properties of transglutaminase. Adv. Enzymol. 38: 109–191.

Folk, J.E. and Finlayson, J.S. 1977. The ε-(γ-glutamyl)lysine cross-link and the catalytic role of transglutaminase. Adv. Protein Chem. 31: 1–133.

Griffin, M. and Wilson, J. 1984. Detection of ε-(γ-glutamyl)lysine. Molecular Cellular Biochem. 58: 37–49.

Griffin, M., Wilson, J., and Lorand, A. 1982. High-pressure liquid chromatographic procedure for the determination of ε-(γ-glutamyl)lysine in proteins. Anal. Biochem. 124: 406–413.

Hurrell, R.F., Carpenter, K.J., Sinclair, W.J., Otterburn, M.S., and Asquith, R.S. 1976. Mechanism of heat damage in proteins. 7. The significance of lysine-containing isopeptides and of lanthionine in heat proteins. Br. J. Nutr. 35: 383–395.

Icekson, I. and Apelbaum, A. 1987. Evidence for transglutaminase activity in plant tissue. Plant Physiol. 84: 972–974.

Ichihara, K. 1990. *Statistics for Bioscience—Practical Technique and Theory*. Nankodo, Tokyo, Japan.

Ikura, K., Kometani, T., Sasaki, R., and Chiba, H. 1980a. Crosslinking of soybean 7S and 11S protein by transglutaminase. Agric. Biol. Chem. 44: 2979–2984.

Ikura, K., Kometani, T., Yoshikawa, M., Sasaki, R., and Chiba, H. 1980b. Crosslinking of casein components by transglutaminase. Agric. Biol. Chem. 44: 1567–1573.

Ishihara, T., Hasegawa, K., and Kawaguchi, T. 1990. *An Introduction of Lotus 1-2-3 to Multivariate Statistical Analysis*. Kyoritsu syuppan, Tokyo Japan.

Kato, A., Wada, T., Kobayashi, K., Seguro, K., and Motoki, M. 1991. Ovomucin-food protein conjugates prepared through the transglutaminase reaction. Agric. Biol. Chem. 55: 1027–1031.

Kimura, I., Sugimoto, M., Toyoda, K., Seki, N., Arai, K., and Fujita, T. 1991. A study on the cross-linking reaction of myosin in kamaboko "suwari" gels. Bull. Jpn. Soc. Sci. Fish. 57: 1386–1396.

Kirchner, J.G. 1978. *Thin-Layer Chromatography*. John Wiley & Sons, Inc., New York.

Kowata, H., Harada, T., Matsukubo T., and Takaesu Y. 1987. Influence of texture of processed foods on parotid salivary secretion and mastication. Nippon Eiyo Shokuryo Gakkaishi 40: 299–305.

Kumazawa, Y., Seguro, K., Takamura, M., and Motoki, M. 1993. Formation of ε-(γ-glutamyl)lysine cross-link in cured horse mackerel meat induced by drying process. J. Food Sci. 58: 1062–1064, 1083.

Kurth, L. and Rogers, P.J. 1984. Transglutaminase catalyzed cross-linking of myosin to soya protein, casein, and gluten. J. Food. Sci. 49: 573–589.

Loewy, A.G. and Matacic, S.S. 1981. Modulation of the ε-(γ-glutamyl)lysine cross-link in cellular proteins. Biochim. Biophys. Acta 668: 167–176.

Margosiak, S.A., Dharma, A., Bruce-Carver, M.R., Gonzales, A.P., Louie, D., and Kuhen, G.D. 1990. Identification of large subunit of ribulose 1,5-bisphosphate carboxylase/oxygenase as a substrate for transglutaminase in medicago sativa l. (alfalfa) Plant Physiol. 92: 88–96.

Motoki, M. and Nio, N. 1983. Crosslinking between different food proteins by transglutaminase. J. Food. Sci. 48: 561–566.

Nio, N., Motoki, M., and Takinami, K. 1985. Gelation of casein and soybean globulins by transglutaminase. Agric. Biol. Chem. 48: 2283–2286.

Nio, N., Motoki, M., and Takinami, K. 1986a. Gelation mechanism of protein solution by transglutaminase. Agric. Biol. Chem. 50: 851–855.

Nio, N., Motoki, M., and Takinami, K. 1986b. Gelation of protein emulsion by transglutaminase. Agric. Biol. Chem. 50: 1409–1412.

Nonaka, M., Sakamoto, H., Toiguch, S., Kawajiri, H., Soeda, T., and Motoki, M. 1992. Sodium caseinate and skim milk gels formed by incubation with microbial transglutaminase. J. Food. Sci. 57: 1214–1218.

Nonaka, M., Tanaka, H., Okiyama, A., Motoki, M., Ando, H., Umeda, K., and Matsuura, A. 1989. Polymerization of several proteins by Ca^{2+} independent transglutaminase derived from microorganisms. Agric. Biol. Chem. 53: 2619–2623.

Nonaka, M., Toiguchi, S., Sakamoto, H., Kawajiri, H., Soeda, T., and Motoki., M. 1994. Changes caused by microbial transglutaminase on physical properties of thermally induced soy protein gels. Food Hydrocolloids 8: 1–8.

Otterburn, M.S. 1983. *Isopeptides: The Occurrence and Signification of Natural and Xenobiotic Crosslinks in Proteins*. ACS Symp. Ser. 234 (Xenobiot. Foods Feeds): 221–232.

Otterburn, M., Healy, M., and Sinclair, W. 1977. The formation, isolation and importance of isopeptides in heated proteins. Adv. Exp. Med. Biol. 86b: 239–262.

Pisano, J.J., Finlayson, J.S., and Peyton, M.P. 1968. Cross-link in fibrin polymerized by Factor XIII. Science 160: 892–893.

Rao, M.A. and Steffe, J.F. 1992. *Viscoelastic Properties of Foods*. Elsevier Applied Science, London and New York.

Sakamoto, H., Harada, T., Matsukubo, T., Takaesu, Y., and Tazaki, M. 1989. Electromyographic measurement of textural changes of foodstuffs during chewing. Agric. Biol. Chem. 53: 2421–2433.

Sato, K., Tsukamasa, Y., Imai, C., Ohtsuki, K., Shimizu, Y., and Kawabata, M. 1992. Improved method for identification and determination of ε-(γ-glutamyl)lysine cross-link in protein using proteolytic digestion and derivatization with phenyl isothiocyanate followed by high-performance liquid chromatography separation. J. Agric. Food. Chem. 20: 806–810.

Schmitz, I., Zahn, H., Klostermeyer, H., Rabbel, K., and Watanabe, K. 1976. On the occurrence of isopeptide bonds in heated milk protein. Z. Lebensm.-Unters. Forsch. 160: 377–381.

Seki, N., Uno, H., Lee, N-H., Kimura, I., Toyoda, K., Fujita, T., and Arai, K. 1990. Transglutaminase activity in Alaska pollack muscle and surimi, and its reaction with myosin B. Bull. Jpn. Soc. Sci. Fish. 56: 125–132.

Signorini, M., Beninati, S., and Bergamini, C.M. 1991. Identification of transglutaminase activity in the leaves of silver beet (*Beta vulgaris* L.) J. Plant Physiol. 137: 547–552.

Singh, H. 1991. Modification of food proteins by covalent crosslinking. Trends in Foods Sci. & Technol. August: 196–200.

Tanaka, H., Nonaka, M., and Motoki, M. 1990. Polymerization and gelation of carp myosin by microbial transglutaminase. Bull. Jpn. Soc. Sci. Fish. 56: 1341.

Tarcsa, E. and Fesus, L. 1990. Determination of ε-(γ-glutamyl)lysine crosslink in proteins using phenylisothiocyanate derivatization and high-pressure liquid chromatographic separation. Anal. Biochem. 186: 135–140.

Tsukamasa, Y., and Sato, K., Shimizu, Y., Imai, C., Sugiyama, M., Minegishi, Y., and Kawabata, M. 1993. ε-(γ-Glutamyl)lysine crosslink formation in sardine myofibril sol during setting at 25°C. J. Food. Sci. 58: 785–787.

Tsukamasa, Y., and Shimizu, Y. 1990. Setting property of sardine and pacific mackerel meat. Bull. Jpn. Soc. Sci. Fish. 56: 1105–1112.

Tsukamasa, Y. and Shimizu, Y. 1991. Factors affecting the transglutaminase-associated setting phenomenon in fish meat sol. Bull. Jpn. Soc. Sci. Fish. 57: 535–540.

Washizu, K., Ando, K., Koikeda, S., Hirose, S., Matsuura, A., Takagi, H., Motoki, M., and Takeuchi, K. 1994. Molecular cloning of the gene for microbial transglutaminse from *Streptoverticillium* and its expression in *Streptomyces lividans*. Biosci. Biotech. Biochem. 58: 82–87.

Whitaker, J.R. 1977. Enzymatic modification of proteins applicable to foods. In *Food Proteins; Improvement through Chemical and Enzymatic Modification*. American Chemical Society, Washington, DC.

Williams-Ashaman, H.G., Notides, A.C., Pabalan, S.S., and Lorand, L. 1972. Transamidase reaction involved in the enzymic coagulation of semen; isolation of γ-glutamyl-ε-lysine dipeptide from clotted secretion protein of guinea pig seminal vesicle. Proc. Natl. Acad. Sci. 69: 2322–2325.

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Transport of Oleic and Acetic Acids from Emulsions into Low Density Polyethylene; Effects on Adhesion with Aluminum Foil in Laminated Packaging

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ABSTRACT

Three test emulsions were prepared by adding oleic acid and/or acetic acid to a fatty food simulant (oil-in-water emulsion) from rapeseed oil, using Tween-80 as emulsifier. These test emulsions and the blank were packed in laminated material and stored for 4 wk at room temperature (21°C). Sorption of oleic acid into LDPE and the rate of acetic acid permeation through LDPE were highest for emulsions containing both oleic and acetic acids. A complete delamination within 4–7 days occurred for all acid-containing test-emulsions, probably caused by formation of a weak boundary layer (WBL).

Key Words: oleic acid, acetic acid, LDPE, laminate, adhesion

INTRODUCTION

LAMINATES OF PAPERBOARD and low density polyethylene (LDPE) have become common packaging materials for liquid foods. This type of packaging provides many advantages over glass, e.g., low weight, sealability and high impact resistance. An important property of LDPE is its good heat sealability, very important in high-speed packaging (Paine and Paine, 1983). LDPE is a good barrier for water but an ineffective gas barrier. For this reason, packaging laminates for use with foods sensitive to oxidation, often contain an Al-oxygen barrier, which also provides a barrier to light. Adhesion between aluminum and LDPE is fairly low. For many applications, adequate adhesion can be achieved by introducing polar groups at the LDPE surface by oxidation. This is accomplished by using a high temperature extrusion coating process, in combination with ozone treatment of the hot extruded film (Hjertberg et al., 1989).

Certain foods can cause adhesion failures between polyethylene and Al foil in packages (Schroeder et al., 1990). Fat-soluble organic substances such as aroma oils, certain food colorants and fats are readily dissolved in nonpolar polymers such as polyethylene and polypropylene (Charara et al., 1992; Figge, 1980; Halek and Luttmann, 1990; Koch et al., 1976; Koch and Figge, 1978; Kwapong and Hotchkiss, 1987; Nielsen et al., 1992). The sorption of citrus aroma oils, especially d-limonene, into LDPE has been reported (Baner et al., 1991; Halek and Meyers, 1989; Hirose et al., 1987; Imai et al., 1990). In our laboratory, organic acids and fatty acids have been shown to cause rapid delamination in LDPE-Al seals (Olafsson et al, 1993 a,b; Olafsson and Hildingsson, 1995). Since delaminated packages would be ineffective from a food-quality point of view, we considered it important to further explore this potential problem.

Liquid foods such as fruit juices and milk, and especially fermented milk products may contain organic acids and free fatty acids. During storage, concentrations of such components may increase due to chemical and/or microbiological activity.

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Our objective was to investigate the delaminating effects of free fatty acids in waterborne fatty food products and possible synergistic interactions between free fatty acids and acetic acid. The study was performed with a food simulant consisting of a stable emulsion of rapeseed oil in water, which provided simultaneous simulation of both acidic and fat containing foods.

MATERIALS & METHODS

Preparation of emulsions

Oil in water emulsions were prepared from refined rapeseed oil (Karlshamn Oil & Fat AB, Sweden), containing <0.1% free fatty acids, by first mixing 10 parts (by weight) of rapeseed oil, or 10 parts of a mixture of 90 parts rapeseed oil and 10 parts oleic acid (Laurodan Fine Chemicals, Malmö) with 0.3 parts of an ethoxylated sorbitan oleate emulsifier (Tween 80, HLB15, ICI Speciality Chemicals, Leatherhead, UK). After adding 90 parts water to the oil phase, the mixture was prehomogenized for 15 min, using a high speed mixer, Ultra Turrax (IKA, Breisgau, Germany). The final emulsification step was performed at room temperature (≈21°C) in a high pressure homogenizer, (Cook & Laguce). (Microfluidizer™ Inc., Newton, MA, USA) at 1000 bar pressure difference with two passes through the homogenizer. Acetic acid (Merck, Darmstadt, Germany) was added after emulsification. The oleic acid and Tween-80 were 99% purity, acetic acid was analytical grade, water was HPLC grade.

The size of droplets in the emulsions was determined using inelastic laser light scattering (Malvern Mastersizer, Malvern Instruments, UK). Emulsions were measured directly after preparation and after 4 wk to determine their stability. The volume mean diameter, d(4,3) and the volume median diameter d(V, 0.5), were calculated from the distributions (Table 1). Evaluation of stability showed that all emulsions except that containing oleic acid and acetic acid could be considered relatively stable. In the two-acid sample a small part of the oil formed larger droplets but the major fraction remained dispersed (the volume median diameter of droplets was unaltered).

Food packaging material

The packaging material was an experimental laminate supplied by TETRA PAK AB (Lund, Sweden). It consisted of: LDPE (12g/m²)/paper (186g/m²)/LDPE (25g/m²)/Al foil (6.5 μm)/LDPE (15g/m²)/LDPE (25g/m²). All polymer layers except the innermost layer, were extrusion-coated at 320°C. The innermost layer was extrusion-coated at 290°C, using ozone treatment. No tie layers were used. The LDPE (Exxon LD 256) had a density of 0.92 g/cm³.

Table 1—Oil droplet sizes in the various emulsions[a]

Emulsion	Day 0 D (4,3)[b] (μm)	Day 0 D (V, 0.5)[c] (μm)	Day 28 D (4,3) (μm)	Day 28 D (V, 0.5) (μm)
Blank emulsion	0,67	0,41	1,26	0,44
Emulsion + C18:1	0,69	0,42	1,18	0,45
Emulsion + HAc	0,84	0,36	1,56	0,45
Emulsion + C18:1 + HAc	0,97	0,45	8,40	0,43

[a] Size was measured on fresh emulsions and after 4 wk storage.
[b] Volume mean diameter.
[c] Volume median diameter.

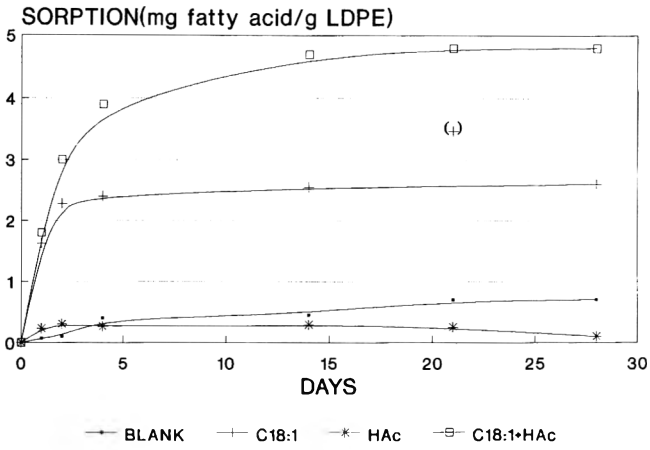

Fig. 1—Fatty acid of $C_{18:1}$ sorbed into inner LDPE layers of a packaging laminate from rapeseed oil-in-water emulsions with or without oleic and/or acetic acid during storage for 4 wk at 21°C.

Test packaging

The material was formed into pouches (12 × 15 cm), by a thermosealer (sealing temperature 190°C; pressure 7 bars; dwell time 5 sec.). Pouches were filled with 50 mL of test emulsions, sealed and stored in an upright position at 21°C. Stock emulsion was used as a blank. Samples were removed after 1, 2, 4, 7, 14, 21 and 28 days. The following measurements were made: (1) amount of sorbed oleic acid in the inner LDPE layer, (2) adhesion between inner LDPE layers and Al foil, (3) presence of oleic acid and acetic acid on the peeled off Al foil and the inner LDPE film by FTIR, and (4) wettability of Al foil (contact angle measurements). In separate experiments permeation of acetic acid through LDPE film was measured using special permeation cells (Hildingsson and Törnell, 1995).

Sorption of fatty acids

The pouches were opened, emptied and interior LDPE surface rinsed with ethanol and Millipore® water. The inner LDPE layers (≈1.0 g) were peeled away from the Al foil and weighed. The plastic film was put into 40 mL pear shaped glass bottles, 30 mL of petroleum ether (analytical grade, Merck) was added and the bottles were allowed to stand 24 hr at room temperature (21°C). A >95% yield was achieved in one extraction. After removing the extracted polymer the petroleum ether was removed by vacuum evaporation (Rotavapor® R-110, Büchi AB, Switzerland). The residue was redissolved in 10 mL of a 5/4 mixture of diethyl ether and ethanol, and the amount of the free fatty acid determined by titration with 0.01 N NaOH in 99.5% ethanol using phenolphthalein as indicator (AOAC.1984). Two replicate measurements were made for each sample.

Adhesion tests

The adhesive strength between Al and inner LDPE layers was measured using a JJ Tensile Testing Machine, model T30K equipped with a 100 N load cell and a recorder, model A 128 (JJ Lloyd Instruments, Southampton, England). A 180° peel test was carried out at a crosshead speed of 50 mm/min as described (Olafsson et al., 1993 a,b). For each sample four strips from each of two pouches were analyzed, that is, eight replicate analyses were made for each emulsion and storage time.

IR analysis

A Fourier Transform Infra Red (FTIR) spectrometer of type Bruker IFS48 (Bruker Analytische Messtechnik Gmbh, Germany) was used, with a nitrogen-cooled MCT detector. For the analysis of Al surfaces a reflection-absorption spectroscopy attachment (RAS Spectretec FT-80) was used, with a fixed angle of incidence of 80°. The spectra of the samples were referred to the reference spectra of the original Al surface. The LDPE film was peeled off and the sample with Al foil was stored in a vacuum desiccator until analysis. Two replicate measurements were made for each sample.

For analysis of LDPE film an attenuated total reflection (ATR) attachment was used. The crystal was a thallium iodide-bromide KRS-5, with angle of incidence 45°. The LDPE layer was peeled off and scanned 200 times to give a sufficiently high resolution spectrum, purging with dry air to remove water vapor. The ATR analyses were made on the side originally facing the aluminum foil. The absorbances of peaks were compared to a reference peak at 2660 cm^{-1}, arising from C-H vibrations, with a baseline at 2420 cm^{-1}, (Colthup et al., 1964). A single measurement was made for each sample.

Contact angle measurement

Contact angle measurements on the Al foil were made using a motor-driven micro-syringe, a video camera monitor and printer (Kober and Wesslén, 1992). A 15 mm strip of material was cut out and the LDPE layer peeled off. The sample was attached on a glass plate, with the Al foil facing up, using a double-sided adhesive tape. By means of micro-syringe a droplet (35–100µl) was placed on the Al foil and the advancing contact angle was recorded. The syringe motor was then reversed and water drawn into the syringe until the edges of the water droplet moved, when the receding contact angle was recorded. The angles were evaluated from video prints. Two samples were taken from each pouch and four measurements, of the advancing and the receding angle, made on each.

Permeation of acetic acid

The rate of permeation of acetic acid through LDPE film was measured at room temperature (21°C), using liquid permeation cells consisting of two continuously stirred glass chambers, each with capacity 55 mL, separated by a 43 µm thick polyethylene film. The permeation area of the plastic film was 19.6 cm^2. The permeation cells were filled with emulsions free from, or containing oleic acid. To one chamber in each cell 3% acetic acid with a small amount of C^{14} labeled acetic acid (Sigma chemical co, St. Louis, USA) was added. The rate of permeation was evaluated by following the increase with time, of the concentration of acetic acid in the receiving chamber. The determination of acetic acid was facilitated by liquid scintillation counting. In these determinations, a 0.5 mL sample of receiving chamber emulsion was mixed with 5 mL scintillation liquid (Ultima gold, Packard, Zürich, Switzerland) in a 6 mL plastic vial. The radioactivity was measured in a liquid scintillation counter (Rackbeta 1219, LKB Wallac, Finland). The permeation coefficient [g mm/m^2 days] was calculated according to:

$$P = \frac{(\text{quantity of permeant})(\text{film thickness})}{(\text{film area})(\text{time})}$$

Statistics

Statistical evaluation of experimental results was made with Minitab® Statistical Software (Minitab Inc., New York, USA).

RESULTS

Sorption measurements

Results showed the sorption of free fatty acid into the inner LDPE layers (Fig. 1). The LDPE layers exposed to the blank emulsion and the acetic acid containing emulsion contained low, almost negligible free fatty acids. In the inner LDPE layers in packages containing emulsion with oleic acid, and emulsion with both oleic and acetic acid, the free fatty acid content increased rapidly and then seemed to approach equilibrium towards the end of the test period. Disregarding the possible effect of free fatty acids in the rapeseed oil, the final values at equilibrium, for sorption of oleic acid were 2.6 mg/g LDPE for the emulsion containing oleic acid and 4.8 mg/g LDPE, for that with both oleic and acetic acids.

Adhesion

Results of the peel tests (Fig. 2) showed that all emulsions containing added carboxylic acids caused delamination between LDPE and Al foil. The packaging materials exposed to the two emulsions containing acetic acid were both delaminated by day four. However, the interlayer adhesion decreased more rapidly in packages containing the emulsion with both oleic and acetic acids. Results also showed that acetic acid and oleic acid be-

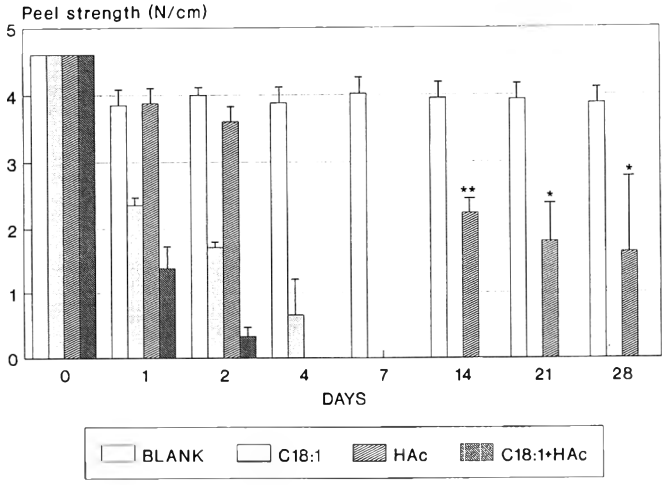

Fig. 2—Strength of adhesion between LDPE and Al foil layers of laminates stored in emulsions containing oleic (1%) and/or HAc (3% in a water) for 4 wk at 21°C. Error bars represent the positive standard deviation of eight replicates.

haved differently. Thus, in the experiment with acetic acid the peel strength, at first completely lost was eventually recovered and reached about half its initial value. A similar recovery did not occur with emulsions containing added oleic acid. The interlayer adhesion for the packaging material exposed to blank solution decreased slightly on day one and remained about constant throughout the study.

Surface analysis by FT-IR

Al foil. In the FT-IR measurements (Fig. 3) of the Al surface, five peaks were examined. These were two close lying peaks near 3000 (cm^{-1}) and peaks at 1605 (cm^{-1}), 1480 (cm^{-1}) and 1430 (cm^{-1}). Aliphatic hydrocarbon absorbs near 3000 (cm^{-1}) (Colthup et al, 1964). The carboxylate ion gives rise to two peaks: a strong peak at 1650–1550 (cm^{-1}) and a weaker peak near 1400 (cm^{-1}) (Colthup et al, 1964). Apart from these, a broad peak at 3800–3000 (cm^{-1}) and a sharp peak at 1000 (cm^{-1}), arising from Al hydroxide were seen in the Al foil exposed to acetic acid. No peaks were observed on the Al foil from the laminate exposed to the emulsion containing oleic acid or to the blank emulsion, at the wave lengths studied. The IR spectra for the Al foil exposed to acetic acid (Fig. 3a) showed on day 4 no carboxylate i.e. acetate was visible on the Al foil. On day 7 three peaks were visible at 1605-, 1480- and 1430 (cm^{-1}). The intensity of these peaks had increased on day 14, 21 and 28. The IR spectra for the Al foil exposed to oleic acid and acetic acid (Fig. 3b) showed on days 4 and 7 no peaks were present, but in the sample studied after 14 days, peaks appeared at 1605- and 1480 (cm^{-1}). The intensity increased with time (after 14, 21 and 28 days). Peaks for aliphatic hydrocarbons also appeared on day 14.

LDPE-film. Three peaks related to the samples were detected in the LDPE film, at 1753 (cm^{-1}), 1715 (cm^{-1}) and 1585 (cm^{-1}). The carbonyl group (C=O) in the carboxylic acids absorbs strongly in the region around 1700 (cm^{-1}), the long chain fatty acids at 1715 (cm^{-1}) and acetic acid at 1745 (cm^{-1}). Carboxylate anion (COO^-) absorbs strongly in the region around 1600 (cm^{-1}) (Colthup et al., 1964; Rugg et al., 1954; Silverstein et al., 1981).

The results of the FTIR measurements for the LDPE film (Fig. 4) showed absorption for the film exposed to the blank emulsion (Fig. 4a) did not increase markedly during the experimental period. The LDPE film exposed to the emulsion containing oleic acid showed a peak at 1715 (cm^{-1}), which increased gradually with time, indicating the presence of fatty acid at the polymer-aluminum interface (Fig. 4b). The film exposed to the emulsion

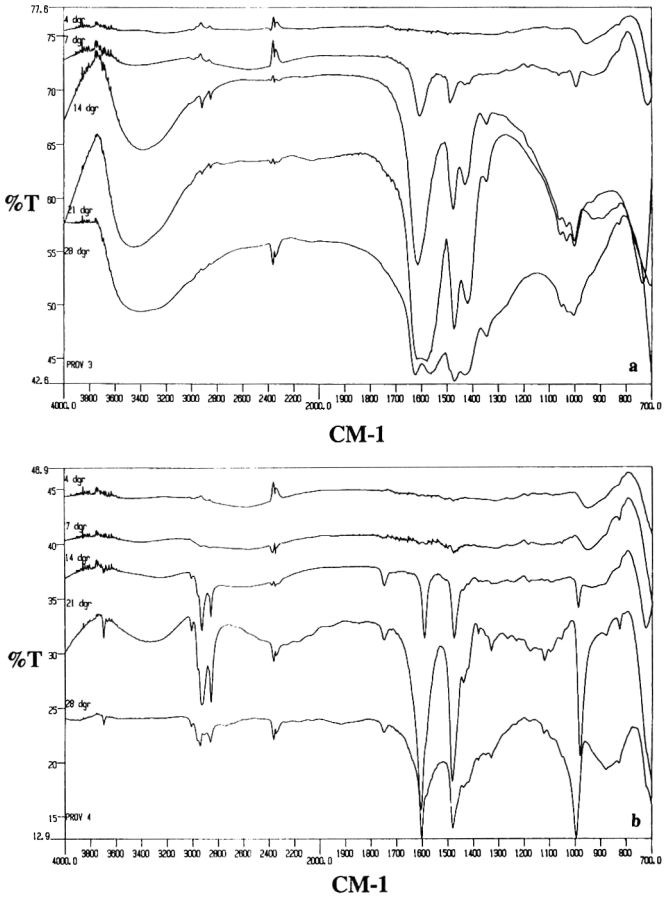

Fig. 3—FTIR (RAS) spectra of Al foil surfaces from packages filled with rapeseed oil in water emulsions, stabilized by Tween 80. (a) Emulsion containing HAc (3% in water); (b) emulsion containing $C_{18:1}$ (1%) + HAc (3% in water). Peaks at 1605 cm^{-1} and 1430 cm^{-1} originate from carboxylate groups [$-COO^-$] on the Al foil.

containing acetic acid (Fig. 4c) absorbed strongly at 1735 (cm^{-1}) and 1585 (cm^{-1}), the intensity increasing rapidly from day 2 throughout the study. This indicated the presence of the carboxylic acid 1735 (cm^{-1}) and a carboxylate (acetate) 1585 (cm^{-1}). The film exposed to the emulsion containing oleic acid and acetic acid (Fig. 4d) absorbed strongly in the same areas.

Contact angle

Contact angle measurements were made to determine the wettability of the Al surface. Two different angles, *advancing* and *receding*, were measured. The contact angle for Al foil in the blank emulsion (Table 2a) remained constant from day 2 throughout the study. The contact angle for Al exposed to emulsions with oleic acid, increased from day 1 to day 28 and those with acetic acid decreased abruptly from day 2 to day 4. In the beginning of storage, the contact angle for Al exposed to oleic and acetic acids were similar to that for Al exposed only to oleic acid. By day 21, an extremely hydrophobic layer appeared and the contact angle reached 143° on day 28.

The receding contact angle measurements (Table 2b) on Al exposed to blank emulsion was almost constant throughout storage. The receding contact angle for foil exposed to oleic increased over time as did the advancing angle. The receding contact angle for Al exposed to acetic acid followed the same pattern as the advancing angle, indicating a steady state was reached on day 4. The receding contact angle for Al foil exposed to oleic acid and acetic acid increased in the beginning of the study and then decreased towards the end of the test period.

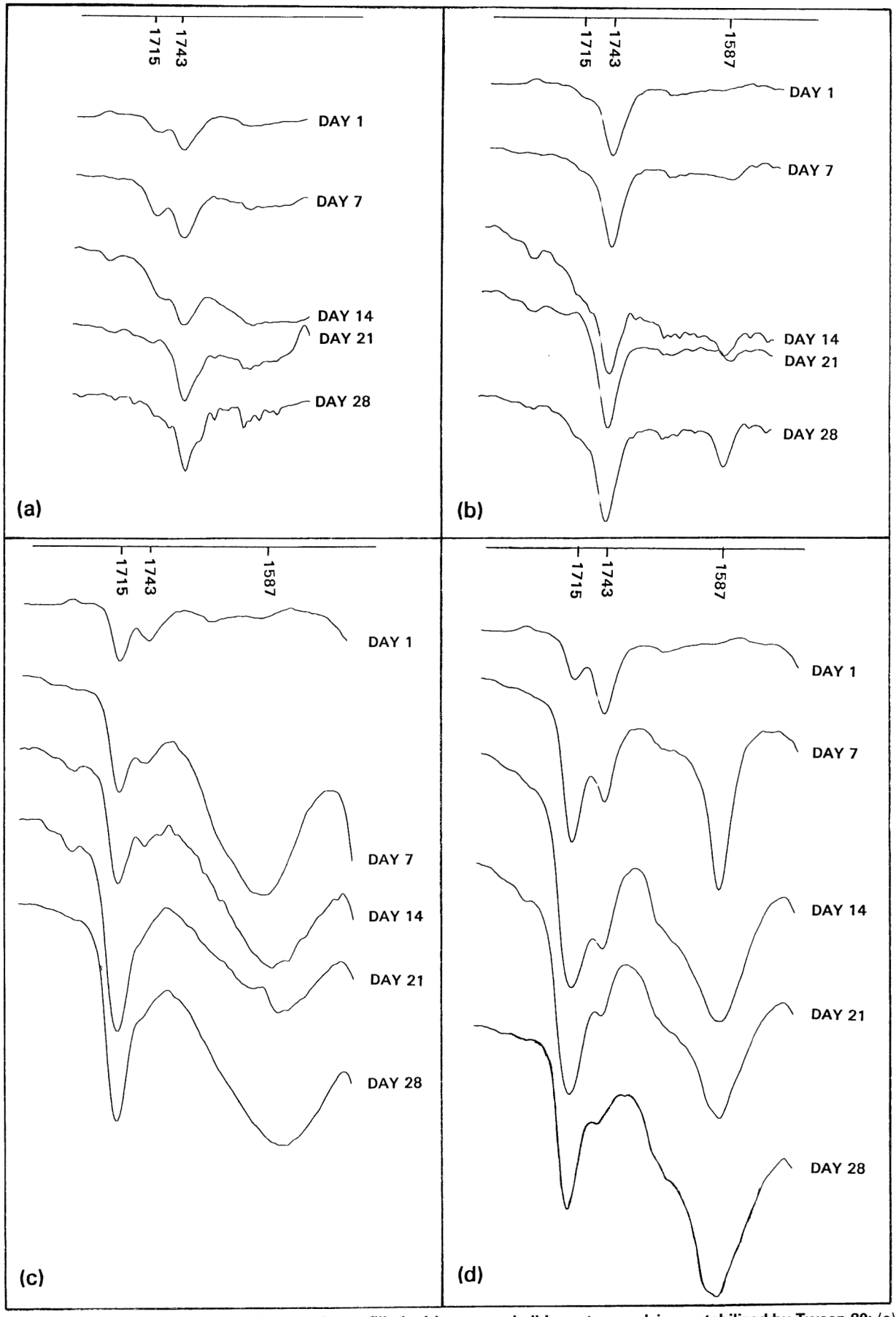

Fig. 4—FTIR (ATR) spectra of LDPE film from packages filled with rapeseed oil-in-water emulsions, stabilized by Tween 80: (a) blank emulsion; (b) emulsion containing $C_{18:1}$ (1%); (c) emulsion containing HAc (3% in water); (d) emulsion containing $C_{18:1}$ (1%) + HAc (3% in water). The peak at 1715 cm^{-1} originates from carbonyl [>C = O] and peaks at 1740 cm^{-1} and 1587 cm^{-1} originate from carboxylate groups [$-COO^-$].

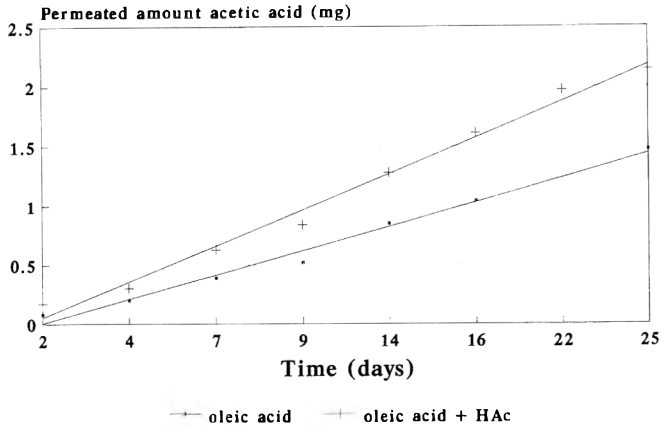

Fig. 5—Permeation of HAc through LDPE from a rapeseed oil in water emulsion with or without $C_{18:1}$ (1%), at 21°C.

Permeation measurements

Results of acetic acid permeation measurements (Fig. 5)showed permeation of acetic acid through LDPE was higher in the emulsion containing oleic acid than in the blank emulsion. In both cases acetic acid permeation rate reached steady state in <2 days.

Table 2—Contact angles for aluminum foil exposed to an oil-in-water emulsion (10:90) stabilized with Tween (3% of the oil phase) with added $C_{18:1}$, HAc or both

a. Advancing contact angle

DAYS	BLANK[a]	$C_{18:1}$	HAc	$C_{18:1}$ + HAc
0	96 ± 1	96 ± 1	96 ± 1	96 ± 1
1	98 ± 2	102 ± 2	97 ± 2	103 ± 2
2	100 ± 3	103 ± 2	95 ± 2*	101 ± 2
4	98 ± 1	104 ± 4	59 ± 3**	102 ± 2
7	100 ± 2*[b]	107 ± 3**	67 ± 5**	104 ± 2
14	100 ± 2*	108 ± 2**	69 ± 15**	105 ± 3
21	100 ± 2*	106 ± 3**	64 ± 7**	120 ± 10**
28	100 ± 2*	113 ± 2**	74 ± 11**	143 ± 7**

b. Receding contact angle

DAYS	BLANK	$C_{18:1}$	HAc	$C_{18:1}$ + HAc
0	71 ± 1	71 ± 1	71 ± 1	71 ± 1
1	65 ± 2	72 ± 1	60 ± 3	68 ± 2
2	69 ± 2**	76 ± 2**	51 ± 6**	72 ± 8
4	67 ± 2*	76 ± 3**	16 ± 3**	81 ± 8**
7	67 ± 2	81 ± 4**	12 ± 4**	83 ± 3**
14	68 ± 2**	89 ± 2**	22 ± 9**	74 ± 9*
21	70 ± 3**	91 ± 2**	26 ± 10**	65 ± 7
28	67 ± 3	96 ± 3**	20 ± 8**	63 ± 4*

[a] Pure emulsion is used as a blank solution. Mean ± SD of eight replicates.
[b] Significance was tested against value on day 1.
* $P < 0.05$.
** $P < 0.01$.

DISCUSSION

WITHOUT OLEIC ACID, the LDPE samples showed negligible acidity, indicating no significant interactions with acetic acid. Whether this increase in sorption of oleic acid depends on interactions between oleic acid and acetic acid is uncertain. After 4 days we observed that a number of larger oil droplets had been formed in the emulsion containing the two acids, indicating an instability. This probably also included a fusion of droplets with the surface, thereby increasing contact between oil and the LDPE. A layer of oil was also seen on the LDPE film when pouches were opened and prepared for measurements. However, sorption of $C_{18:1}$, in the emulsion containing HAc, was higher on day 2. Thus, the formation of larger droplets may have started earlier without being noticed.

Permeation of acetic acid through LDPE was enhanced in presence of oleic acid. In this case the emulsion was stable, i.e. no visible oil droplets were observed since these studies were performed under gentle stirring. Acetic acid is probably absorbed as a dimer, which partly reduces the polar nature of the permeant. The small quantities sorbed (4.8 mg/g LDPE) were hardly adequate to cause enough swelling to change barrier properties of the plastic film. However, the sorbed fatty acid may assist in transport of acetic acid through the polymer matrix by forming hydrogen bonds with the permeating acid. This would allow it to move from one binding site to another through the film. Also FT-IR (ATR) scans of the LDPE film indicated the presence of carboxylic acids originating either from oleic acid or HAc and an acid salt, probably an acetate.

Rapid penetration of both acetic acid and/or oleic acid caused a delamination in as little as 2 days. The rate of delamination decreased in the following order: $C_{18:1}$ + HAc ≥ HAc > $C_{18:1}$. However, adhesion of the laminate exposed to acetic acid partly recovered after about 2 wks storage. This effect has been reported (Olafsson et al., 1993 a,b) and was explained by the formation of Al acetate and various hydrated Al oxides and hydroxides on the Al surface. These salts of HAc are believed to partly replace initial adhesive materials between the two layers. FT-IR (RAS) measurements of the Al surface indicated a chemical reaction between acetic acid and Al foil, forming a salt (acetate). This peak first appeared on day 7 and showed a gradual increase over time. Moreover, contact-angle measurements showed that when Al foil was exposed to acetic acid an extremely hydrophilic layer was formed, capable of forming strong interactions with water (H-bonds).

The adhesion did not recover, for the laminate exposed to $C_{18:1}$ and acetic acid. Delamination probably occurred when oleic acid and acetic acid penetrated the LDPE layer and accumulated in the polymer-Al interface, creating a weak boundary layer (WBL). Contact angle measurements showed increased wettability of the surface of Al foil exposed to acetic acid, due to formation of an acetate. Acetate formation was also detected with FTIR on LDPE film and on Al foil. On foil exposed to $C_{18:1}$ and acetic acid a carboxylate was detected on LDPE film and on foil, although somewhat less than on those exposed to acetic acid alone. However, contact angle measurements indicated an extremely hydrophobic layer on the Al surface. This means that in spite of carboxylate formation, wettability decreased because of the penetrating fatty acid. This may explain why no recovery in adhesion occurred. Interestingly, the emulsion alone caused no delamination. Triglycerides are sorbed by LDPE, but this did not seem to affect interlayer adhesion (Figge, 1980). We therefore concluded that the delaminating effect was caused by free fatty acids rather than by triglycerides.

The colloidal food simulant we used was an oil-in-water emulsion stabilized with ethoxylated sorbitan oleate (Tween-80). The emulsifier covers the oil/water interface its nonpolar tail directed into the oil and its polar head-group directed towards the water. The oil is dispersed in droplets 0.7–1 μm in diameter and a droplet size of ≤1 μm is required to obtain a stable emulsion (Bergenståhl and Claesson, 1989). In this system, oleic acid would be predominantly dissolved in the oil droplets and the acetic acid predominantly dissolved in the water-phase. The oleic acid in the oil core of the emulsion droplets would be in equilibrium with the oleic acid dissolved in the aqueous phase including oleic acid in mixed micelles. The adsorbed surface layers would be in equilibrium with the dissolved material in the aqueous phase. The diffusion of the acetic acid in the water phase towards the LDPE film should be relatively fast compared to the fatty acids. In an adhesion experiment with a similar laminate, a 3% wt/wt solution of acetic acid in water delaminated the LDPE-Al in 3 to 4 days (Olafsson et al., 1993 a,b). That was about the same time required for the acetic acid in the emulsion to delaminate. Oleic acid can be transported in the emulsion in oil-droplets, in micelles containing solubilized oleic

acid, or by diffusion of molecularly dissolved oleic acid in the aqueous phase.

Many different products have been used to simulate fatty foods: n-heptane is recommended by the Food & Drug Administration, USA; HB 307 (a mixture of triglycerides) and olive oil are recommended by the Bundesgesundheitsamt, Germany, and olive oil is recommended by the European Community. Various milk products, mayonnaise, various sauces and dressings are emulsions. The emulsion we used can be made from highly purified oils of known composition. Also the intensive treatment in homogenization causes cell disruption and results in a fairly sterile emulsion. It does not require heat treatment or preservatives, and it is stable at normal pH provided droplets are small (about 1 μm in diameter). It also simulates almost any complex food matrix. A disadvantage, however, is its instability under highly acidic conditions.

List of Abbreviations

ATR:	attenuated total reflection
FTIR:	Fourier transform infrared spectrometry
KRS-5:	thallium iodide-bromide crystal
LDPE:	low density polyethylene
MCT detector:	mercury cadmium tellurium detector
RAS:	reflection-absorption spectroscopy
Tween-80:	ethoxy (20) sorbitan oleate
WBL:	weak boundary layer

REFERENCES

AOAC, 1984. *Official Methods of Analysis.* Assoc. Official Analytical Chemists, Arlington, VA.

Baner, A.L., Kalyankar, V., and Shoun, LH. 1991. Aroma sorption evaluation of aseptic packaging. J. Food Sci. 56: 1051–1054.

Bergenståhl, B. and Claesson, P.M. 1989. Surface forces in emulsion. In *Food Emulsions* K. Larsson and S. Friberg (Ed.), p. 41–96. Marcel Dekker, Inc., New York.

Charara, Z.N., Williams, J.W., Schmidt, R.H., and Marshall, M.R. 1992. Orange flavor absorption into various polymeric packaging material. J. Food Sci. 57: 963–6, 972.

Colthup, N.B., Daly, L.H. and Wiberley, S.E. 1964. *Introduction to Infrared and Raman Spectroscopy.* Academic Press, New York and London.

Cook, E and Laguce, A.P. 1985. U.S. Patent 4,533,254.

Figge, K. 1980. Migration of components from plastic packaging materials into packed goods—test methods and diffusion models. Prog. Polym. Sci. 6: 187–252.

Halek, G.W. and Luttmann, J.P. 1990. Sorption behavior of citrus-flavor compounds in polyethylenes and polypropylenes: Effects of permeant functional groups and polymer structure. Ch. 18, in *Food and Packaging Interactions II,* S.J. Risch and J.H. Hotchkiss (Ed.), p. 203–212. ACS Symposium Series no. 473, American Chemical Society, Washington, DC.

Halek, G.W. and Meyers, M.A. 1989. Comparative sorption of citrus flavor compounds by low density polyethylene. Packag. Tech. and Sci. 2: 141–146.

Hildingsson, I. and Tornell, B. 1995. Synergy in sorption and transport of acetic acid in low density polyethylene. In *Food and Packaging Materials—Chemical Interactions,* Ackerman et al. (Ed.). Royal Soc. Chem. Proceedings of the International Symposium held in Lund, Sweden, June 8–10, 1994.

Hirose, K., Harte, B.R., Giacin, J.R., Miltz, J., and Stine, C. 1987. Sorption of d-limonene by sealant films and effect on mechanical properties. Ch. 3, in *Food and Packaging Interactions,* J.H. Hotchkiss (Ed.), p. 28–41. ACS Symposium Series no. 365, American Chemical Society, Washington, DC.

Hjertberg, T., Sultan, B.-Å., and Sörvik, E.M. 1989. The effect of corona discharge treatment of ethylene copolymers on their adhesion to aluminum. J. Appl. Polym. Sci. 37: 1183–1195.

Imai, T., Harte, B.R., and Giacin J.R. 1990. Partition distribution of aroma volatiles from orange juice into selected polymeric sealant films. J. Food Sci. 55: 158–162.

Kober, M. and Wesslén, B. 1992. Amphiphilic segmented polyurethans as surface modifying additives. J. Polym. Sci. 30: 1061–1070.

Koch, J., and Figge, K. 1978. Bestimmung der Fettlässigkeit von Lebensmitteln. II. Fette, Seifen, Anstrichmittel. 80: 158–161.

Koch, J., Robinson, L., and Figge, K. 1976. Bestimmung der Fett-lässigkeit von Lebensmitteln. I. Fette, Seifen, Anstrichmittel. 78: 371–377.

Kwapong, O.Y. and Hotchkiss, J.H. 1987. Comparative sorption of aroma compounds by polyethylene and ionomer food-contact plastics. J. Food Sci. 52: 761–763, 785.

Mannheim, C.H., Miltz, J., and Letzter, A. 1987. Interaction between polyethylene laminated cartons and aseptically packed citrus juices. J. Food Sci. 52: 737–740.

Nielsen, T.J., Jägerstad, I.M., Öste, R.E., and Wesslén, B.O. 1992. Comparative absorption of low molecular aroma compounds into commonly used food packaging polymer films. J. Food Sci. 57: 490–492.

Olafsson, G. and Hildingsson, I. 1995. Sorption of fatty acids into low density polyethylene and its effect on the adhesion between layers in laminated packaging material. J. Agric. Food. Chem. 43: In press.

Olafsson, G., Jägerstad, M., Öste, R., and Wesslén, B., and Hjertberg, T. 1993a. Effects of different organic acids on the adhesion between polyethylene film and aluminum foil. Food Chem. 47: 227–233.

Olafsson, G., Jägerstad, M., Öste, R., and Wesslén, B. 1993b. Delamination of polyethylene and aluminum foil layers of laminated packaging material by acetic acid. J. Food Sci. 58: 215–219.

Paine, F.A., and Paine 1983. *A Handbook of Food Packaging.* Blackie & Son Ltd London.

Rugg, F.M., Smith, I.J., and Bacon, R.C. 1954. Infrared spectrophotometric studies of polyethylene II. oxidation. J. Polym. Sci. 13: 535–547.

Schroeder, M.A., Harte, B.R., Giacin, J.R., and Hernandez, R.J. 1990. Effect of flavour sorption on adhesive and cohesive bond strength in laminations. J. Plastic Film & Sheeting 6: 232–246.

Silverstein, R.M., Bassler, C.G. and Morill, T.C. 1981. *Spectrometric Identification of Organic Compounds.* Wiley, New York.

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Erratum Notice

•*J. Food Sci.* (Vol. 60, No. 1) pages 40-41, 54, Particle size and mixing time effects on sensory and physical properties of low-fat, high-moisture pork frankfurters by A.D. Small, J.R. Claus, H. Wang, and N.G. Marriott. On page 40, the last paragraph under MATERIALS & METHODS, Product manufacture contains some errors. The sentence should read: "The smokehouse schedule was: 20 min at dry bulb 51.7°C and wet bulb off, 15 min at dry bulb 65.6° and wet bulb 55.6°C with smoke, 15 min at dry bulb 76.7°C and wet bulb 65.6°C with smoke, steam-cooked until internal temperature was achieved (ca 4 min) and showered 5 min." Please correct accordingly.

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Guidelines for the Preparation and Review of Nutrition-Related Papers

by the IFT Nutrition Division

☐ THE NUTRITION DIVISION of the Institute of Food Technologists has prepared the following guidelines for use by authors in preparing nutrition-related manuscripts for publication in *Journal of Food Science*, as well as for use by reviewers when evaluating the suitability of such manuscripts for publication.

I. Papers concerning essential nutrients should emphasize the properties of such nutrients as they occur in foods consumed by humans. Examples would include:

A. Studies of the chemical and/or physical properties and stability of nutrients.

B. Studies of dietary components or processing treatments that affect the bioavailability of nutrients as they exist in foods commonly consumed by humans.

II. Papers concerning nutrition-related studies of nonessential dietary constituents (e.g., various forms of dietary fiber, lipids, etc.) should be relevant to human nutrition. The following technical details should be addressed:

A. Proper description of the chemical composition, important chemical and physical properties, and source of key experimental and dietary materials must be provided.

B. Attention should be given to potential differences in digestibility and/or gastrointestinal kinetics of dietary ingredients (especially dietary fiber and protein) in dry vs hydrated form.

III. Papers describing research conducted using either animal models or human subjects is potentially appropriate for publication in *Journal of Food Science*. The following should be considered:

A. The use of an animal model should be justified as to its relevance to human nutrition. Studies based on animal experimentation that does not adequately model the human situation would not be acceptable.

B. Studies using animal models should include a statement that the research was reviewed and approved by the Institutional Animal Care and Use Committee at the researcher's institution or that the research complied with guidelines described in the *Guide for the Care and Use of Laboratory Animals* (NRC, 1985).

C. Studies using human subjects should include a statement that the research was reviewed and approved by the Institutional Review Board at the researcher's institution or that it was in compliance with the Helsinki Declaration of 1975, revised in 1983.

D. The composition of animal diets should be fully described and justified with respect to the requirements of the species used.

E. In experiments in which the chemical composition and physical properties of dietary constituents may have important nutritional effects (e.g., dietary fiber or lipid sources), such information must be provided in adequate detail.

F. The source and strain of experimental animals should be provided.

IV. *In vitro* methods proposed as models for the study of nutritional processes (e.g., bioavailability) must be fully justified with respect to their physiological relevance and correlation with *in vivo* methods.

V. Papers dealing with the nutritional properties of ingredients or foodstuffs that are not typically consumed by humans (or are potential human foodstuffs) would not ordinarily be acceptable.

VI Papers dealing specifically with basic biochemical functions of nutrients would not ordinarily be acceptable.

VII. Papers concerning nutritional aspects of toxicology are potentially acceptable. However, toxicological studies should be relevant to constituents commonly found in human diets, including those indigenous to foodstuffs, microbial products, compounds produced as a result of chemical reactions occurring during food processing and/or storage, and relevant environmental contaminants. In addition, studies of the nutritional aspects of toxicology should be relevant to questions pertaining to human nutrition. It is essential that papers describing toxicology studies provide complete information regarding dietary composition and experimental protocols.

Reference

NRC, 1985. *Guide for the Care and Use of Laboratory Animals*, NIH Publ. 85-23. Prepared by Commission on Life Sciences, Natl. Res. Council, Natl. Institutes of Health, Bethesda, Md.

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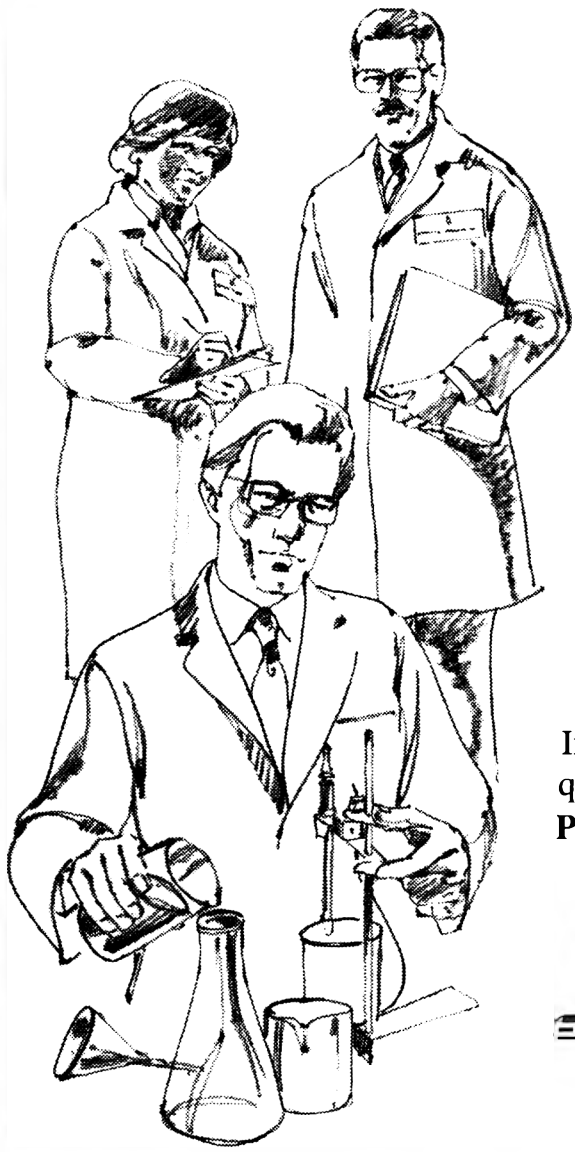

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