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Electronic Startup for JFS

W E ARE BEGINNING to move into the electronic age with our Journal. If you have had a manuscript accepted in the past few months you already know we-'re asking for the final revised, edited version on disk in electronic format (Word Perfect, DOS, ASCII files). In this issue you will find suggestions and detailed instructions on preparing the final disk version (p. iv, this issue). Of course, we still need one hard copy with all changes incorporated to rely on for backup, should anything unexpected happen to the disk version.

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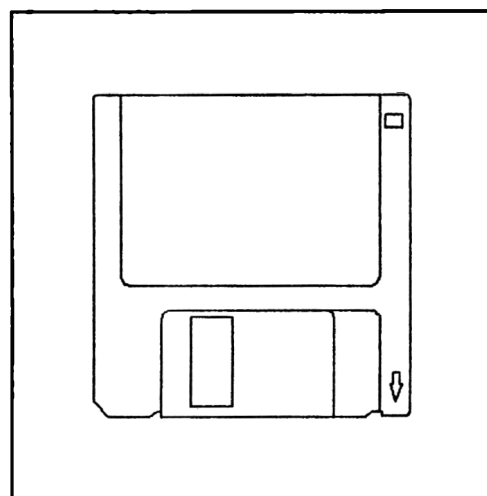
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Predicting Milk Shelf-life Based on Artificial Neural Networks and Headspace Gas Chromatographic Data

B. VALLEJO-CORDOBA, G.E. ARTEAGA, and S. NAKAI

ABSTRACT

The usefulness of artificial neural networks (ANN) for milk shelf-life prediction by multivariate interpretation of gas chromatographic profiles and flavor-related shelf-life was evaluated and compared to principal components regression (PCR). The training set consisted of dynamic headspace gas chromatographic data collected during storage of pasteurized milk (input information for the neural network used to make a decision) and its corresponding shelf-life (prediction or response). ANN had better predictability than PCR. A standard error of the estimate of 2 days in shelf-life resulting from regression analysis of experimental vs predicted values indicated a high predictability of ANN.

Key Words: milk, shelf-life, predictability, artificial neural networks, principal components

INTRODUCTION

SHELF-LIFE OF MILK indicates how long the product will remain acceptable once it has been processed. Many tests have been developed to estimate milk shelf-life ranging from bacterial enumeration to metabolite detection (Bishop and White, 1986). One approach to shelf-life determination is the use of gas chromatographic (GC) profiles of milk volatiles as prediction indicators. Studies at the University of Minnesota involving a limited number of milk samples have shown this approach had promise (San Buenaventura et al., 1991). An optimized method for dynamic headspace gas chromatography (DH-GC) was useful for detecting and identifying volatiles developed during milk shelf-life (Vallejo-Cordoba and Nakai, 1993). Multivariate interpretation of headspace GC profiles and flavor-related shelf-life was required to derive shelf-life prediction models. High correlation between flavor-related shelf-life and volatiles detected by DH-GC was obtained by principal components regression (PCR) (Vallejo-Cordoba and Nakai, 1994a).

In PCR, which is basically a regression on principal components and a parametric statistical technique, certain assumptions must be met. An example of this technique was presented by Draper and Smith (1981). PCR is not efficient in analyzing nonlinear systems (Borggard and Thodberg, 1992). Often transformations of the data are required to achieve closer approximations to the assumptions. However, transformations of Y affect least squares considerations and are often used for the wrong reasons (Zaar, 1984). Although utilization of nonlinear models is not popular in flavor studies, nonlinear models frequently provide a better fit of biological phenomena (Aishima and Nakai, 1991).

For nonlinear problems, neural networks are the most promising alternative technique (Borggard and Thodberg, 1992). Neural networks are useful when no exact mathematical information is available (Eerikainen et al., 1993). Another advantage of a neural network over a rule-based model is that, if the process under analysis changes, new examples can be added and the neural net can be trained again. This is much easier than determining new models or rules. Moreover, no underlying statistical assumptions are made on the behavior of the data (Wythoff et al., 1990). Artificial neural networks (ANN) are mathematical models of biological neural systems. Although they are gross simplifications of actual physical cognitive processes, application of these models has indicated that ANN have strength and weaknesses in the same areas as humans (Wythoff et al., 1990). Neural networks are not known for precision; if precision is not as important as speed, a neural net may be useful (Lawrence, 1991). Studies on the use of ANN in relating sensory perceptions to physical measurements further support this view (Thai and Shewfelt, 1991). Galvin and Waldrop (1990) predicted at a sensory evaluation symposium that neural networks would allow automation where complex unstructured tasks involving human insight, creativity and judgment are required.

Neural networks learn from examples through iteration, without requiring *a priori* knowledge of relationships between variables under investigation (Eerikaineen et al., 1993). Each example includes both inputs (information used to make a decision) and patterns (prediction or response). ANN tries each example in turn using the inputs to calculate answers which it compares to provided patterns. When it is wrong, ANN corrects the network by making changes to internal connections. The trial and error process continues until the network outputs are in good agreement with patterns to a certain specified level of accuracy.

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Once the network is trained and tested, new inputs are given to produce predictions. Connections link the inputs to a layer of cells called hidden neurons; these hidden neurons are, in turn, linked to outputs by a second set of connections. Thus the network consists of three layers of processing units: (a) A layer of inputs which serves as distributor of inputs to the network. (b) Eight layers of hidden units which perform a weighted sum of the inputs followed by a nonlinear sigmoid transformation with the connections referred to as weights. (c) A layer of output units, often just one, which again performs a weighted sum of the inputs. The output units are receiving inputs from the hidden units as well as directly from the input units. The weights are the adjustable parameters. The learning rules (such as the back propagation algorithm) are used for comparing actual and desired output of the network, thereby adjusting the weights of the network in order to reduce error (Borggard and Thodberg, 1992).

A schematic representation and explanation of how these layers are interconnected was presented by Arteaga and Nakai (1993). Detailed discussions of ANN theory have been reported (Jansson, 1991; Lawrence, 1991; Wythoff, 1993).

Thus, the design of a neural network consists of the following steps (Lawrence, 1991): (a) Define the problem (determine whether the ANN is going to predict, generalize or recognize. (b) Choose information on which the ANN will base its tasks. (c) Organize the data as facts. A fact is a collection of inputs coupled with the correct output(s). (d) Train the network by repeatedly presenting a set of facts to the network. The network takes in each input, makes a guess as to the output, checks this guess against the output supplied, and makes corrections to internal connections when its guess is incorrect. This process is repeated for each fact in turn until the network learns the facts well enough to be useful (outputs that are within a preset range of accuracy). (e) Test the network, which is essentially the same as training it, except that the network is shown facts it has never seen before and no corrections are made when the network is wrong. If results are good, the network is ready to be used. If not, more or better data should be gathered, or the network should be redesigned.

Our objective was to investigate the

ability of ANN to predict flavor-related shelf-life based on volatiles in GC profiles and compare with that of principal components regression (PCR).

MATERIALS & METHODS

PASTEURIZED MILK was sampled during refrigerated storage at 4°C until termination of shelf-life, as determined by sensory evaluation described by Vallejo-Cordoba and Nakai (1994a). Subsamples were incubated at $24 \pm 1°C$ for 18 hr prior to detection of volatiles by dynamic headspace GC (Vallejo-Cordoba and Nakai, 1993). Several volatiles consisting mainly of aldehydes, ketones, and alcohols were identified in good-quality milk. Not only increased peak areas of the compounds already present appeared in poor-quality milk, new volatiles were also detected, including esters.

Chromatograms were divided into 47 ranges which included all peaks in the chromatograms that had been reported by Vallejo-Cordoba and Nakai (1994a). Ratios of each of 46 area ranges to the area of the internal standard (standardized peak areas) were calculated and the log transformations were used in further data processing. A Lotus spreadsheet was constructed consisting of 134 facts or cases containing GC data and shelf-life assessed by sensory tests.

ANN and PCR were used and compared for their predictability. ANN was applied using the commercial software Brainmaker (California Scientific Software, Grass Valley, CA). A three-layer network was designed with the 46 log transformations of standardized peak areas from each chromatogram as inputs (input layer); the output (output layer) was sensory-based shelf-life in days. After preliminary trials, it was found that eight hidden layers gave the best results. Using 20 or 30 hidden layers resulted in unreliable prediction during testing of the network. Increasing the number of hidden neurons resulted in overfitting of the data (Wythoff, 1993). To estimate the true predictability of ANN with a new set of data, cross-validation was used. The data were divided into two sets. The first set (113 cases) was used for training and the second data set (21 cases) was used for testing the network.

PCR was applied by using commercial Systat software (Wilkinson, 1990). In PCR, the independent variables were the first 30 principal components, and the dependent variable was flavor-based

shelf-life in days. To compare the predictive ability of ANN and PCR, regression analysis was carried out for experimental vs predicted shelf-life, and the squared correlation (r^2) and the standard error of the estimate (SEE) were calculated. To gain insight into the distribution of the error across the entire data set, the standard error of prediction (SEP) was calculated for the total set, and for groups assigned to 'good', 'marginal', and 'poor' defined by an arbitrary specified shelf-life range.

RESULTS & DISCUSSION

THE SHELF-LIFE PREDICTABILITY of ANN was superior to PCR as shown by scattergrams of experimental vs predicted shelf-life for the complete data sets (Fig. 1 and 2). Regression statistics by these two techniques further confirmed that ANN predicted shelf-life more accurately as shown by a standard error of the estimate (SEE) of 2 days (Table 1). Unlike a previous analysis of a similar data set where the logarithmic transformation of shelf-life was used in PCR (Vallejo-Cordoba and Nakai, 1994a), in our study regression analysis was carried out on the non-transformed shelf-life data. Results could be interpreted more straightforward without having to convert back to the original data without transformation. Another advantage of ANN is that, when a new observation is available, it can simply be added to the data set before training and testing is carried out again. Prediction of shelf-life with a cross-validation data set in ANN was less accurate than with the whole data set as shown by a much larger standard error of the estimate (Table 1). Examination of the scattergram formed by the cross-validation data set revealed five outlying observations (Fig. 1). Regression statistics after elimination of those data points improved correlation of the data, as indicated by a higher coefficient of determination ($r^2 = 0.82$), and increased predictive ability as indicated by a lower standard error of the estimate (SEE = 3.0). However, even with elimination of these outlying observations, the predictive ability of ANN with cross-validation was not as high as that with the total set. The preferred method to validate a model is through the collection of new observations. However, sometimes this is not feasible or practical and cross-validation or data splitting is a reasonable alternative for testing true predictability (Neter et al., 1990). Sac-
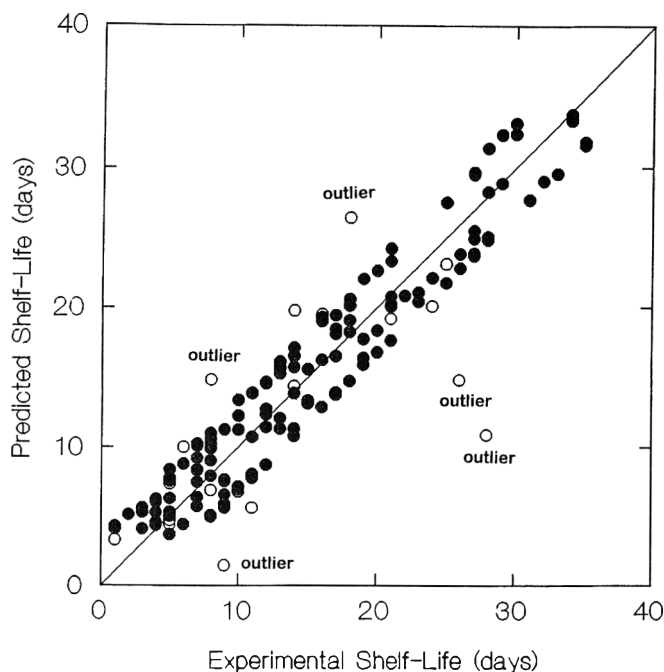
Fig. 1—Predictability of the trained artificial neural network (ANN) for milk shelf-life. The ANN consisted of 46 variables (GC areas) as input neurons, 8 hidden neurons and shelf-life (days) as output neuron. (●) The complete set of 134 milk samples was used for training and prediction, (○) cross-validation set (n = 21). The solid line represents a perfect match between experimental and predicted values.
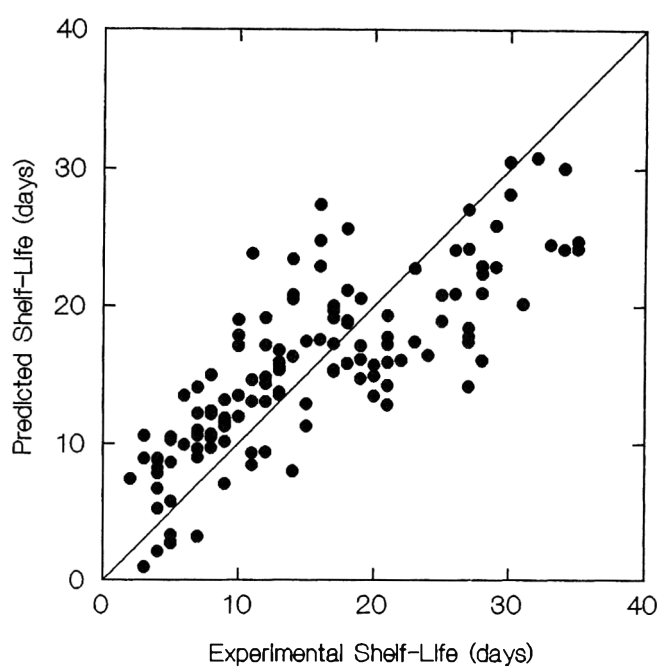
Fig. 2—Predictability of principal components regression (PCR) for milk shelf-life. PCR consisted of the first 30 principal components extracted from 46 variables (GC areas) as the independent variables and shelf-life (days) as the dependent variable in forward stepwise selection. (●) The complete set of 134 milk samples was used for training and prediction. Cross-validation was not performed. The solid line represents a perfect match between experimental and predicted values.

rificing part of the data by not including it during training has its disadvantages, particularly when the training data set is not sufficiently large so that representative information is present during training. Another consideration, that had to be made, was any bias present in the training data set. For example, the data set used may have an uneven distribution with many more cases with longer shelf-life than those with shorter shelf-life.

ANN provided a standard error of the prediction (SEP) of about half of that for PCR in the total data set (Table 2). Shelf-life was more accurately predicted for the larger group of samples classified as 'good' than for the groups of samples classified as 'marginal' or 'poor' (Table 2). According to Wythoff (1993), networks would likely perform better in cases with greater frequency of appearance in the training data because the error minimization would be biased toward it. One way to overcome this is to divide the training data into separate 'bags', one for each group and to sample from each bag with equal likelihood. However, this does not make very efficient use of training data (Wythoff, 1993). Although bias in the training data set towards better performance with samples classified as 'good' is a possi-

Table 1—Comparison of regression statistics of artificial neural networks (ANN) and principal components regression (PCR) for prediction of milk shelf-life[a]

Method	All samples (n = 134)		Cross-validation (n = 21)	
	r²	SEE	r²	SEE
ANN	0.92	2.25	0.50 (0.82)[b]	5.34 (3.0)[b]
PCR	0.62	4.21	—	—

[a] Squared correlation coefficient (r²) and standard error of the estimate were calculated for the predicted versus the experimental shelf-lives. ANN and PCR were computed using 46 variables (GC areas) with shelf-life as the response. ANN consisted of 46 input neurons, 8 hidden neurons and shelf-life as the output neuron. PCR consisted of the first 30 principal components as the independent variables and shelf-life as the dependent variable in forward stepwise regression.

[b] Squared correlation coefficient (r²) and standard error of the estimate calculated after elimination of five outlying observations.

bility, better predictive ability for this group could also be interpreted as being more homogeneous. Samples in the 'good' quality group were tested for volatiles at a time when bacterial metabolic activity was lower than for those samples in the 'marginal' or 'poor' quality groups. During periods of high metabolic activity, volatile production could have been the result of different types of spoilage, thus resulting in wider variance of the chromatographic profiles for the same shelf-life.

Indeed, the presence of different off-

Table 2—Comparison of the standard error of prediction of artificial neural networks (ANN) and principal components regression (PCR) for shelf-life prediction of milk samples assigned to different quality groups[a]

Method	Standard error of prediction (%)			
	Total	Good	Marginal	Poor
ANN	15.0	10.4	22.1	32.0
PCR	28.0	17.0	38.4	76.0

[a] Milk samples with different shelf-life ratings were assigned to three quality groups: 'good' (shelf-life of 15 days or more, n = 60); 'marginal' (shelf-life of 8 to 14 days, n = 42); and 'poor' (shelf-life of 1 to 7 days, n = 32).

flavors was reported for samples of poor quality as detected by a sensory panel (Vallejo-Cordoba and Nakai, 1994b). The fact that prediction early in milk shelf-life (samples grouped as 'good') was more accurate than closer to the end of shelf-life (samples grouped as 'poor' or 'marginal') has notable practical implication since shelf-life should be predicted immediately after milk processing. Milk shelf-life prediction appears to be an example where techniques such as ANN which can deal with imprecise or noisy data might be most useful. Although ANN has not been fully adopted by the food industry, this technique has multiple applications in flavor-related studies. According to Eerikainen et al. (1993) neural networks are particularly

suitable as "software sensors" for difficult-to-measure parameters and for solving nonlinear problems.

CONCLUSIONS

ARTIFICIAL NEURAL NETWORKS (ANN) were useful for predicting milk shelf-life when applied to GC profiles to correlate with sensory-based shelf-life. ANN had better predictive ability than principal components regression (PCR). ANN is more amenable for quality control situations since new observations can simply be added to the data set for training or testing (prediction) of the neural net. Unlike ANN, when new observations are added to the data set in PCR, principal components have to be calculated before regression analysis is applied.

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Continuous Supercritical Fluid Processing of Anhydrous Milk Fat in a Packed Column

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ABSTRACT

A continuous supercritical carbon dioxide processing system was designed, built and operated to investigate its performance for fractionation of anhydrous milk fat (AMF). Packed columns showed mass transfer efficiencies five times greater than a spray column. Short- and medium-chain fatty acids were concentrated in the extract fractions and their amounts decreased as separation pressure increased. The ratio of unsaturated to saturated fatty acids in the raffinate fraction was 0.68 compared to 0.52 in the original AMF. The proportions of low-melting triglycerides in the range −42 to 15°C were greater in the extract fractions (44–72%) compared to the original AMF (29%).

Key Words: supercritical fluid, extraction, milk fat, fractionation

INTRODUCTION

INCREASING CONCERNS over dietary cholesterol, saturated fat and calorie consciousness have resulted in increases in the national surplus of anhydrous milk fat (AMF) (Rizvi et al., 1989; Williams and Lyones, 1989). This has also been caused by increases in low-fat dairy products such as 1% and 2% fat milk and yogurt. The flavor and mouthfeel of AMF are preferably to any other fat, but its physical and rheological properties, particularly its low spreadability at refrigeration temperatures, make AMF less preferred by consumers. The separation of AMF into fractions with more preferable physico-chemical properties, a more favorable unsaturated to saturated fatty acid ratio and reduction of cholesterol would help increase its markets. Modifications of AMF by hydrogenation (Martine, 1982), interesterification (Sreenivasan, 1978), blending with other edible fats (Vovan and Riel, 1973), short-path distillation (Arul et al., 1988) and melt crystallization (Fjaervoll, 1970) have been reported, but with limited success.

The food industry has applied supercritical fluid extraction for several difficult separation problems (Rizvi et al., 1986a, b). The possibility of fractionating AMF by supercritical carbon dioxide (SC-CO₂) has been reported by several researchers (Kaufmann et al., 1982; Shishikura et al., 1986; Arul et al., 1987; Rizvi et al., 1989; Bradley, 1989), but they have utilized batch systems. However, economically productivity cannot be optimized using a batch type operation.

The shortest processing time would invariably be realized in a continuous SC-CO₂ system. Also, little study has been made of the mass transfer and hydrodynamic performance of continuous extraction devices for extraction of AMF. A few studies were devoted to the measurement and prediction of phase equilibria of AMF in SC-CO₂ (de Haan, 1991; Yu et al., 1992).

Our first objective was to provide information on mass transfer and hydrodynamic performance of a continuous countercurrent SC-CO₂ extraction column. Three processing parameters: reflux, recycle, and temperature gradient in the extraction column, were evaluated for their influence on extraction efficiency for AMF. Our second objective was to evaluate selected physico-chemical properties of the fractions obtained.

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MATERIALS & METHODS

AMF

Commercial grade butter was purchased from the Cornell Dairy Store and converted into AMF by melting at 60°C and filtering through Whatman No. 1 filter paper under vacuum. It was subsequently stored at −20°C for future use.

System and operating conditions

A continuous supercritical fluid separation system was designed, built and operated to investigate its performance for fractionation of AMF (Fig. 1). The system consisted of an extraction section, a recycle loop, a reflux loop, and sections for adsorption, desorption and separation. The system was rated for a pressure of 345 bar. The extraction column (EC) was a 61 cm contacting height and 1.75 cm I.D. stainless steel tube rated to 1,379 bar.

EC was operated in a countercurrent mode as a spray column (with no internals) and as a packed column using wire spirals and knitted mesh packings at 40°C/241 bar for determination of performance characteristics of the extraction column (Table 1). The superficial velocity of CO₂ was held constant at 23.5 cm/min, while the superficial velocity of AMF was varied from 0.27 to 0.85 cm/min.

Processing parameters

In order to investigate the effect of temperature gradient in the EC, recycle, and reflux on extraction of AMF, a EC packed with knitted mesh was operated at 40°C/241 bar. The nominal velocities were CO₂ 20.6 cm/min and AMF 0.42 cm/min. When a temperature gradient was used, the extraction pressure was 241 bar, and the temperatures (°C) were bottom 40, middle 51, and top 60. For the recycle operation, the extraction temperature and pressure were 40°C and 241 bar, and the ratio of recycle to raffinate was 12.3. Reflux operation was at 40°C/241 bar. The reflux ratio was 1.3 and the reflux chamber was 40°C/137 bar.

Fractionation

AMF was fractionated in four stages by separation at different temperatures and pressures. AMF was pumped into EC at 40°C/241 bar and contacted countercurrently with the SC-CO₂. The SC-CO₂ along with the dissolved AMF was separated into four fractions (S1, S2, S3, S4) by temperature and pressure. These were drawn off periodically, quantified, and analyzed for fatty acids and physico-chemical properties. Fractionation conditions were 60°C/241 bar, 80°C/207 bar, 80°C/172 bar and 60°C/69 bar. The raffinate fraction (R) was taken from the bottom of EC, weighed, and analyzed.

Analysis

AMF and its SC-CO₂ fractions were analyzed for cholesterol and fatty acids on a GC fitted with a flame ionization detector (HP 5890, Hewlett Packard, USA). Carrier gas was helium at 1.5 mL/min. Cholesterol was analyzed by a modified method of Lynch and Barbano (1988). Sterols were derivatized to form trimethylsilyl esters which were analyzed using a capillary column coated with SE-30 (Chrompack Co., The Netherlands). Temperatures were oven 250, injector 270, and detector 300°C. Injection volume of sample was 3.8 μL. 5-α cholestane (Sigma Chemical Co., USA) was used as internal standard.

Fatty acids were converted to methyl esters (AOCS, 1989) and analyzed using a capillary column, 15 m × 0.25 mm, Durabond-225 (J & W Scientific Co., USA). Oven temperature was held at 60°C for 2 min, then increased at 4°C/min to 220°C and held 10 min. The temperatures
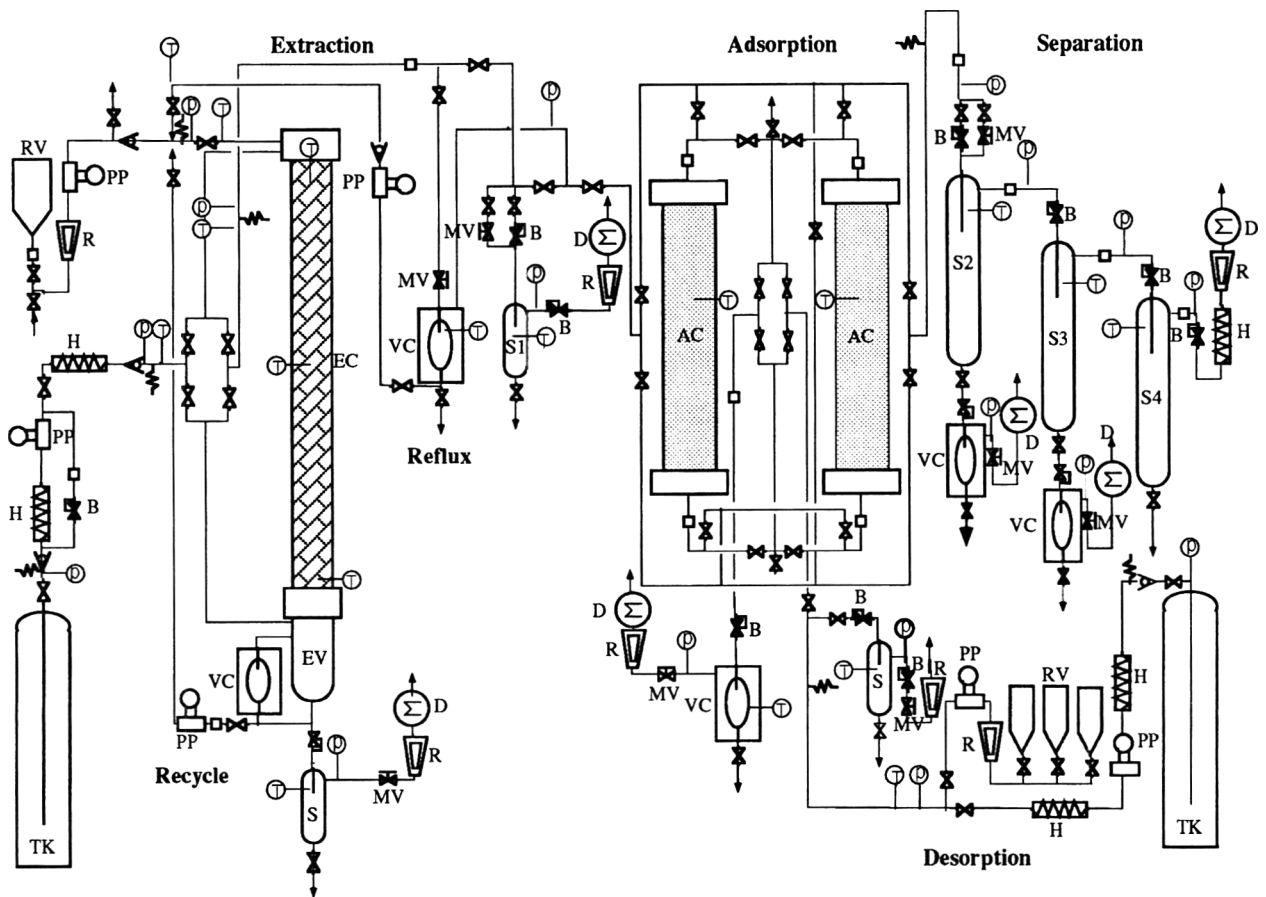
Fig. 1—Schematic diagram of continuous supercritical fluid fractionation system (AC: adsorption column, B: back pressure regulator, D: dry test meter, EC: extraction column, EV: extraction vessel, H: heat excharger, MV: metering valve, P: pressure reading, PP: high pressure pump, PR: pressure regulator, R: rotameter, RV: AMF reservoir, T: temperature reading, TK: carbon dioxide cylinder, V: on/off valve, VC: view cell).

Table 1—Packing characteristics

Type	Wire spirals	Knitted mesh
Material	Stainless steel 316	Stainless steel 316
Dimension (mm)	2.29 × 4.45 × 4.45	—
Surface area (cm²/cm³)	19.5	19.2
Void fraction	0.83	0.95

of the injector and detector were 200 and 240°C, respectively. Injection volume of sample was 2 μL.

Thermal analyses of AMF and its SC-CO₂ fractions were performed using a differential scanning calorimeter (DSC) (Perkin-Elmer, USA, Model DSC-1) according to the procedure of Norris et al. (1971). The variation in solid triglycerides content with temperature was calculated from DSC melting thermograms (Norris et al., 1971).

Theory

Mass transfer efficiency is described by the number of theoretical stages (N) and the height equivalent to a theoretical stage (HETS) (Treybal, 1980). For the extraction of AMF using SC-CO₂, the flow rates of both SC-CO₂ and AMF vary through the column length, especially at high solvent-to-feed ratios. This is due to the change in amounts of AMF present in the column. At the extraction conditions, AMF contained a considerable amount of CO₂, and notable amounts of AMF were dissolved in the SC-CO₂. However, SC-CO₂ was in large excess and its flow rate remained fairly constant. If the solvent-to-feed ratio varied over the column length, then the operating line would be curved.

To account for the change in AMF flow rate, Raj et al. (1993) developed a modified version of Martin's equation (1963) as shown in Eq. (1).

$$N = \frac{\log\left[1 + y_N\left(\dfrac{2L_{N+1} + V_0(1-m) - V_N(1+m)}{2m(y_N V_N - X_{N+1}L_{N+1})}\right)\right]}{\log\left[\dfrac{m(V_0 + V_N)}{2L_{N+1} + V_0 - V_N}\right]} \qquad (1)$$

where, y_N = mass fraction of triglycerides in SC-CO₂ exiting the (n) th stage, L_{N+1} = mass flow rate of AMF feed (g/hr), V_0 = mass flow rate of SC-CO₂ (g/hr), m = equilibrium distribution coefficient of triglycerides, V_N = mass flow rate of the extract which includes CO₂ (g/hr), x_{N+1} = mass fraction of triglycerides in the AMF feed.

Equation (1) was used to calculate N, and HETS for AMF extraction was calculated from Eq. (2).

$$HETS = \text{Packed column height/N} \qquad (2)$$

RESULTS & DISCUSSION

Performance of extraction column

A practical method for process design and scaleup is to determine HETS based on phase equilibrium measurement and pilot scale data. HETS of AMF extraction for various contacting devices were calculated at different superficial velocities of AMF on two different packings and in a spray column (Fig. 2). The spray column was less efficient for mass transfer than the packed columns, as the HETS for spray columns ranged from 290 to 420 cm. This was because AMF fell through the spray column at a faster rate, leading to shorter residence time, and there was less area exposed for mass transfer (Rathkamp et al., 1987; Lahiere and Fair, 1987). For the packed columns with knitted mesh and wire spirals these values were 45 to 75 cm. There was no difference in HETS values between the packed
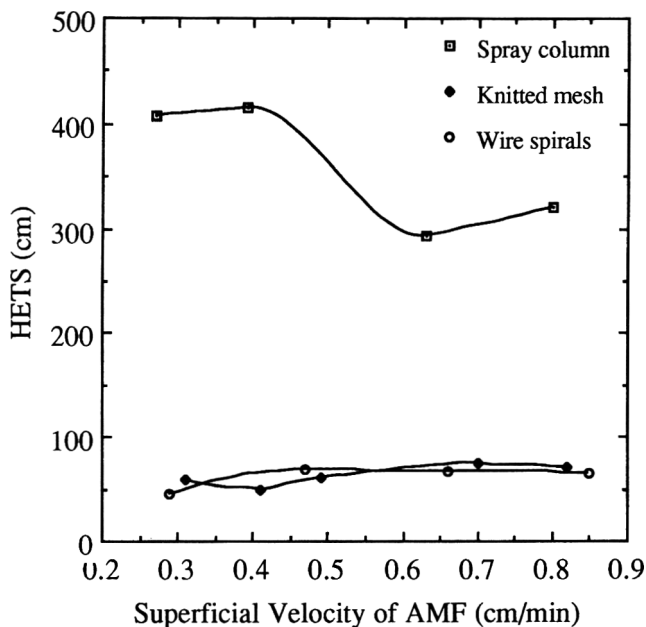
Fig. 2—Efficiency of different column internals for extraction of AMF at 40°C/241 bar calculated by an equation of Raj et al. (1993).

Table 2—Effects of processing parameters on extraction of AMF at 40°C/241 bar

Operation	Original AFM	No treatment	Temp. gradient (40-60°C)	Recycle (R=12.3)	Reflux (R=1.3)
S/F (g/g)	—	48	48	46	49
AMF feed (g/hr)	—	53	54	55	52
CO₂ feed (g/hr)	—	2580	2630	2540	2570
Extracts					
Triglycerides yield (wt%)	—	66	56	59	43
Chol. yield (wt%)	—	71	61	60	47
Chol. changes (%)	—	+8.3	+7.6	+1.8	+7.7
Fatty acids (mol%)					
C4-C8	17.6	20.8	23.4	19.6	24.8
C10-C12	6.1	7.2	7.2	7.0	7.5
C14-C18	76.2	71.8	69.2	73.2	67.5
Unsaturated	26.3	23.6	21.4	24.0	21.0
Saturated	49.8	48.1	47.8	49.1	46.4
Unsat/sat	0.52	0.49	0.44	0.49	0.45
Raffinates					
Triglycerides yield (wt%)	—	34	38	42	56
Chol. yield (wt%)	—	25	38	39	53
Chol. change (%)	—	−26.9	−11.6	−1.8	−5.6
Fatty acids (mol%)					
C4-C8	—	3.3	5.0	9.2	8.3
C10-C12	—	3.9	4.6	4.9	4.8
C14-C18	—	92.6	90.3	85.8	86.7
Unsaturated	—	37.2	36.1	32.7	34.2
Saturated	—	55.3	54.2	53.1	52.5
Unsat/sat	—	0.67	0.66	0.61	0.65

This was because the increase of AMF velocity increased backmixing that tended to lower efficiency (Seibert and Moosberg, 1988). De Haan (1991) also observed that the HETS for the methylnaphthalene-hexadecane-CO₂ system increased as nominal velocity of AMF increased.

However, the HETS values for given systems and packings were almost constant over a given range of nominal velocity of AMF. This was likely due to compensating effects of the increase of interfacial area, turbulence favorable to mass transfer, and increases in backmixing that tend to lower efficiency (Rathkamp et al., 1987; Lahiere and Fair, 1987; Seibert and Moosberg, 1988). Rathkamp et al. (1987) also reported a fairly constant HETS value of 6.7 cm for the isopropanol-water-CO₂ system in a packed column with 6.4 mm raschig rings at a nominal velocity of AMF of 2.5 to 8.9 cm/min with constant nominal velocity of CO₂ of 2.2 cm/min at 40°C/102 bar.

Effect of processing parameters on extraction of AMF

The effects of temperature gradient, recycle and reflux were compared (Table 2) on extraction of AMF EC packed with knitted mesh at 40°C/241 bar. A temperature gradient in EC has been used in fractionation of fish oil esters for better separation of ω-3 fatty acids. By increasing temperature at the top of the column which decreased the density of CO₂, the solubility of triglycerides decreased from the bottom to the top creating internal reflux. The triglycerides yield decreased by 15% compared to no temperature gradient due to density changes associated with the gradient. This led to a decrease in cholesterol yield of 14% compared to no temperature gradient. However, the cholesterol concentration in the extract decreased by only 8% (compared to no temperature gradient) as seen in fatty acid compositions. The extract phase became enriched with short-chain (C4–C8) fatty acids which have a high affinity for cholesterol.

Recycling part of the raffinate is a method used to increase contact time of the raffinate phase. Recycling some of the settled raffinate back to EC may then improve extraction efficiency (Treybal, 1980). On recycling part of the raffinate, triglycerides and cholesterol yields in the extract decreased by 10 and 15%, respectively. This was because the raffinate, which was depleted of cholesterol and lighter triglycerides, diluted the incoming AMF of these substances and they could not be stripped easily. The cholesterol concentration in the extract decreased by 78%.

Refluxing part of the extract is a method used to increase product purity in many chemical separations (Treybal, 1980). The refluxing substances flow downward in the column, washing out heavy components from uprising gas streams. With reflux operation, triglycerides and cholesterol yields decreased by 34 and 33%, respectively. This was because part of the extract was precipitated at reflux conditions, was pumped back and combined with incoming AMF feed. The cholesterol concentration in the extract decreased by only 7% compared to no refluxing because the cholesterol became concentrated in the SC-CO₂ phase.

More short- and medium-chain (C10–C12) fatty acids were extracted by reflux operation because the lighter triglycerides-rich precipitate at reflux condition was combined with incoming feed. High concentrations of long-chain (C14–C18) fatty acids and ratio of long-chain unsaturated/saturated fatty acids were obtained during the no-treatment operation. Most of the short- and medium-chain fatty acids were stripped from the raffinate. This correlated with the higher yield of triglycerides in the extract.

From the results none of the above approaches was likely to provide unique products in terms of fatty acid compositions. Hence a multi-stage separation process for AMF fractionation was investigated.

Fatty acid compositions of AMF and its SC-CO₂ fractions

AMF was fractionated by an extraction and four stage separation using SC-CO₂ at 40 to 80°C and 241 to 69 bar. The fatty

columns. They both yielded lower HETS values and were more efficient than the spray column. Results indicated that the packing increased mass transfer efficiency more than 5-fold compared to the spray column. Lahiere and Fair (1987) also reported that mass transfer efficiency of the packed column, in which a liquid was a continuous phase, was five times greater than that of a spray column. The packing surface increased the tortuosity, thus increasing the residence time of AMF as well as providing a high interfacial area for mass transfer.

The effect of superficial velocity of AMF on mass transfer efficiency was also determined (Fig. 2). There appeared to be a slight decrease in mass transfer efficiency (increase of HETS) of the packed column as nominal velocity of AMF increased.

Table 3—Fatty acid compositions (mol%) of AMF and its SC-CO₂ fractions

					61	
S/F (g/g)					61	
Extract loading (wt%)					1.3	
Triglycerides recovery (%)					101	

	AMF	R	S1	S2	S3	S4
Temperature (°C)	—	40	60	80	80	60
Pressure (bar)	—	241	241	207	172	69
pCO₂(kg/m³)	—	873	779	608	503	153
Triglycerides yield (wt%)	—	19.5	11.8	36.1	17.8	15.4
C 4:0	6.8	1.0	3.9	7.5	10.9	10.6
C 6:0	3.5	0.7	2.4	4.2	5.2	5.5
C 8:0	1.5	0.4	1.1	1.7	2.0	2.2
C10:0	3.2	1.2	2.5	3.4	3.5	4.0
C12:0	3.6	1.9	3.1	3.7	3.7	4.4
C14:0	11.4	8.4	10.8	11.8	11.7	12.7
C14:1	0.8	0.6	0.9	0.9	0.8	0.8
C15:0	1.0	0.9	1.0	1.0	1.0	1.1
C16:0	28.9	28.5	29.5	29.3	28.9	29.0
C16:1	1.3	1.3	1.4	1.3	1.2	1.2
C18:0	12.0	18.4	13.4	10.8	9.6	8.9
C18:1	22.5	32.2	25.8	20.8	18.2	16.9
C18:2	2.7	3.4	3.1	2.5	2.2	1.8
C18:3	0.3	0.4	0.4	0.3	0.3	0.3
SCFA*(C4-C8)	11.8	2.2	7.5	13.6	18.2	18.4
MCFA*(C10-C12)	6.8	3.1	5.6	7.2	7.3	8.4
LCFA*(C14-C18)	81.2	94.6	86.7	79.1	74.3	73.0
Unsaturated	27.8	38.2	31.7	26.1	23.0	21.2
Saturated	53.4	56.4	54.9	53.0	51.3	51.8
Unsat/Sat	0.52	0.68	0.58	0.49	0.45	0.41

*SCFA: short chain fatty acids, MCFA: medium chain fatty acids; LCFA: long chain fatty acids.
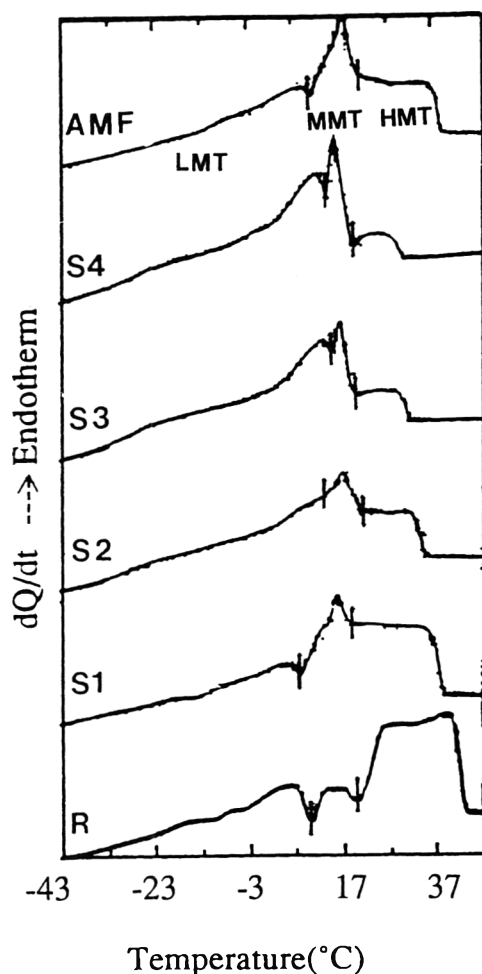
Fig. 3—Melting thermograms of AMF and its SC-CO₂ fractions (LMT: low-melting triglycerides, MMT: medium-melting triglycerides, HMT: high-melting triglycerides).

acid compositions of AMF and its SC-CO₂ fractions were compared (Table 3). Short- and medium-chain fatty acids were concentrated in the extract fractions (S1-S4) and their amounts decreased as separation pressure increased. Considerable amounts of long-chain fatty acids occurred in the raffinate fractions (R) and their amounts decreased as separation pressure decreased. Long-chain unsaturated fatty acids were concentrated in the raffinate fractions while long-chain saturated fatty acids remained fairly constant. The ratio of unsaturated/saturated fatty acids in the raffinate fraction was 0.68 compared to 0.52 in the original AMF.

Thus SC-CO₂ fractionation of AMF provides the possibility of altering fatty acid compositions in fractions by concentrating short-chain fatty acids in extract fractions and maximizing unsaturated fatty acids in raffinate fractions. Fractionation of AMF using SC-CO₂ is based on its triglyceride molecular size (Arul et al., 1987). Though there were discrete fatty acid composition differences among the fractions, fractionation of fatty acids on the basis of molecular size of triglycerides was not sharp, with all fatty acids occurring in all fractions.

Melting thermograms of AMF and its SC-CO₂ fractions

Thermal properties of AMF and its SC-CO₂ fractions were examined by DSC to assess the contribution of different triglycerides to overall physical characteristics (Fig. 3). AMF began to melt above −43°C with maximum melting at 17°C. The high-melting triglycerides appeared between 20 to 38°C as a shoulder on the main peak. All thermograms had sharp upper temperature limits but lower temperature limits were less well defined. The major peak in each curve was attributed to the principal latent heat effect associated with melting triglycerides. In the raffinate fraction the magnitude of the effect was reduced since less triglycerides had solidified. All melting thermograms except that for the raffinate fraction showed the highest rate of melting in the range of 15 to 18°C. This was associated with temperatures at which apparent specific heat value was highest, considering both sensible and latent heat changes (Lewis, 1987). The raffinate fraction showed an exothermic transition at 10°C in the melting thermogram, i.e. dips below the baseline which were probably due to a polymorphic transition from less stable to more stable crystalline forms (Norris et al., 1971).

All the fractions showed 3 melting zones in the melting thermograms: a low-melting zone as minor peak (essentially a shoulder), an intermediate-melting zone represented by the major peak, and a high-melting zone as a broad shoulder. The major differences in melting thermograms of AMF fractions are readily explained by the proportions of triglycerides in each zone (Table 4). The proportions of low-melting triglycerides (LMT) in the temperature range of −42 to 15°C were greater in the extract fractions (S2-S4) ranging from 44 to 72% compared to 29% in the original AMF. The raffinate fractions had high proportions of high-melting triglycerides (HMT) (75%) in the range of 21 to 44°C compared to 38% in the original AMF. Medium-melting triglycerides (MMT) were low in all fractions by 7 to 30% for the range of 9 to 22°C compared to 33% in the original AMF. This decrease was due to a shift from MMT to LMT in the extract fractions and from MMT to HMT in the raffinate fraction. Interestingly, HMT proportions in the range of 19 to 36°C for the extract fractions (S2-S4) were observed to be small ranging from 26 to 12% compared to 38% in the original AMF.

Solid triglycerides content of AMF and its SC-CO₂ fractions

Variations in solid triglycerides content with temperature for AMF and its SC-CO₂ fractions were observed (Fig. 4). The solid triglycerides content decreased sigmoidally as temperature increased. The solid triglycerides contents of all fractions were markedly different from the original AMF. As expected, at any

Table 4—Melting characteristics of AMF and its SC-CO2 fractions

Sample	Low-melting triglycerides Temp. range (°C)	Area (%)	Medium-melting triglyceride Temp. range (°C)	Area (%)	High-melting triglycerides Temp. range (°C)	Area (%)
AMF	-21/10	29	10/20	33	20/38	38
R	-20/11	18	11/21	7	21/44	75
S1	-13/9	20	9/20	26	20/40	54
S2	-42/15	44	15/22	30	22/36	26
S3	-42/15	70	15/20	19	20/32	11
S4	-29/14	72	14/19	16	19/30	12
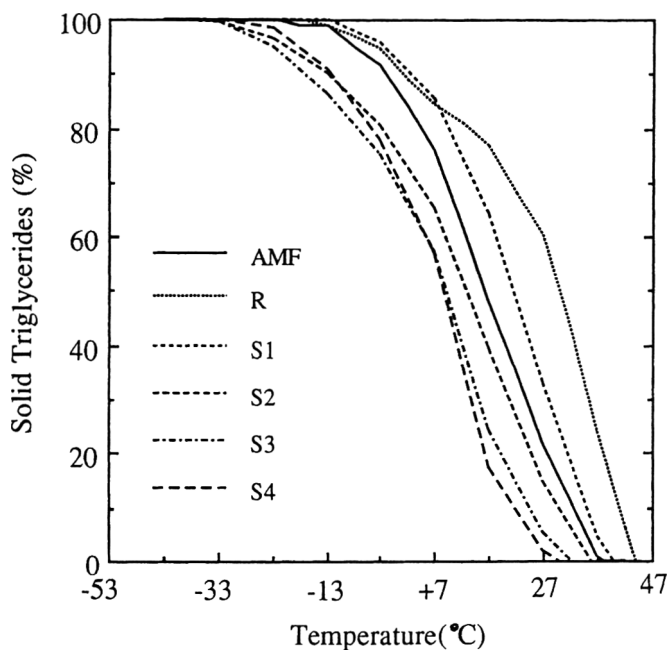
Fig. 4—Solid triglycerides content of AMF and its SC-CO2 fractions.

temperature the solid triglycerides content increased in the order: extract fractions, AMF, the raffinate fraction. This showed good agreement with the trend observed in the proportion of each triglycerides. For example, at 17°C the solid triglycerides content in the extract fractions (S4–S2) was 17 to 39% compared to 48% in the original AMF. The behavior of the solid triglycerides content of the raffinate fraction was comparable with those found in pastry fats made with hydrogenated animal and vegetable fats with typical melting points up to 44°C (Rajah, 1986). This gives them the temperature tolerance for use in layering of doughs in puff pastry. The solid triglycerides content is the most important parameter determining hardness and spreadability of AMF. The curves (Fig. 4) can give a general indication of the suitability of each fraction for a particular purpose in formulated foods. The low-melting fraction can be used in bakery products, confectionery and ice cream, and in milk via recombination (Rajah, 1986).

CONCLUSION

A CONTINUOUS SUPERCRITICAL CO$_2$ processing system enabled assessment of the feasibility of using such separation systems for fractionation of AMF. Specific processing conditions provided tailored AMF fractions, and improved unsaturated/saturated fatty acid ratio/specific functionalities which led to a potential new dairy products. This work should also be useful in the design of high pressure processes for fractionation of AMF and similar dairy fractions.

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Retention of Diacetyl in Milk during Spray-Drying and Storage

A. SENOUSSI, E.D. DUMOULIN and Z. BERK

─────────── ABSTRACT ───────────

Spray-drying of milk in a Leaflash dryer was studied in relation to volatile aroma loss in drying and during storage of the powders Diacetyl was used as the volatile model, and the contribution of the various milk constituents to retention was determined. Proteins retain more diacetyl than does lactose or fat. Conditions leading to a powder with more amorphous lactose result in higher diacetyl loss during drying. The rate of diacetyl loss during storage depended on relative humidity and on the T $-$ T$_x$ parameter and was strongly enhanced by crystallization of amorphous lactose.

Key Words: diacetyl, glass transition, lactose, milk, spray-drying

INTRODUCTION

STRUCTURAL CHANGES affecting bulk and surface properties of the dry mass are known to strongly influence the rate of volatile aroma loss during drying and subsequent storage of spray-dried food powders. The most important of such changes are crystallization and glass transition phenomena. In milk powder, the component most likely to undergo these kinds of structural changes is lactose.

In lactose powders obtained by spray-drying or by lyophilization of solutions, the amorphous form usually predominates (Walstra and Jenness, 1984; Luquet, 1985). This is because the solid structure is fixed very rapidly either by fast drying, in very hot air (spray-drying) or by quick freezing (lyophilization). The amorphous state is a nonequilibrium state, and may undergo change at a rate depending on temperature, water content, etc . . . At temperatures above glassy transition, more or less rapid change from amorphous to rubbery, liquid or crystallized states occurs (Karel, 1990; Blond and Simatos, 1991; Roos and Karel, 1991a,b). At room temperature, crystallization of amorphous lactose occurs at relative humidity $\approx$42%, corresponding to water content of 5-6% (Flink and Karel, 1972; Adrian and Lepen, 1987; Slade and Levine, 1991). The rate of crystallization depends on the difference between storage temperature (T) and the glassy transition point (T$_g$), following the WLF equation (Roos and Karel, 1992). The methods for studying crystallization and state of lactose include X-ray diffraction (Briggner et al., 1994), NIR (Vuataz, 1988) and differential scanning calorimetry (Roos and Karel, 1991a; Slade and Levine, 1991).

The retention of a given volatile component during spray-drying is largely affected by selective diffusion of water vs volatile substances. Above a critical concentration, the diffusion of water alone is observed and organic volatiles are retained (Flink and Karel, 1970; Flink and Labuza, 1972; Chirife et al., 1973). However, in crystalline solids the principal mechanism of volatile substance retention is adsorption on the surface. The adsorption of diacetyl on lactose is considerable and depends on the physical state of the sugar, the α- and β-crystalline forms having adsorption capacities higher than that of amorphous lactose (Nickerson and Dolby, 1971).

Physical structure is also highly relevant to storage stability of foods in powder form. In systems containing lipids, the protective action of the solid matrix may be lost when crystallization occurs. Thus, methyl linoleate encapsulated in a

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lactose-gelatin support is rapidly oxidized when the initially amorphous lactose undergoes crystallization (Shimada et al., 1991). Glassy transition in milk powders accelerates agglomeration and caking (Roos and Jouppila, 1993).

Leaflash drying, a spray-drying technique used in our work, has been previously described and studied as to its suitability for encapsulation of aromas (Bhandari et al., 1992; Senoussi et al., 1994). Our objective was to study the retention by milk of diacetyl, as a model for volatile aroma substances, both during the course of drying and subsequent storage of the powders. The contribution of various components of milk to volatile retention was examined.

MATERIALS & METHODS

Dryer

The dryer used for all experiments was the Leaflash 100 spray-dryer (Rhône-Poulenc, Aubervilliers, France) (Bhandari et al., 1992, Clement et al., 1994). This atomizer is a specific pneumatic nozzle where the pressurized gas is the hot drying air. Hot air (pressure 1.4 bar) is injected with an helicoïdal swirl and accelerated into a convergent duct before impacting the liquid for atomization and drying. Feed is metered into the dryer by a peristaltic pump. Since in the Leaflash the liquid is simply propelled through the central head, atomization of viscous liquids (up to 600 mPa.sec) is easier. The drying air is electrically heated and controlled up to 450°C. In our experiments the wall of the drying chamber of the Leaflash was thermostated at 60°C. Maximal evaporative capacity is 7 kg/hr, and the experimental mean residence time, defined as the elapsed time between entrance of liquid in the atomizer and the first appearance of dry powder in the product collector, is 5 sec. Operating conditions were similar for all experiments: inlet air. 250 $\pm$ 2°C: outlet air, 100 $\pm$ 2°C; air flow rate, 70 m³/hr.

Preparation of powders

Lactose powder was obtained by spray-drying a concentrated lactose solution in the Leaflash. The solution was prepared from α-lactose monohydrate (Sigma Chemical Co., St. Louis, MO; containing 2% β-lactose) at 50°C. After addition of diacetyl (Sigma) at 0.5% of the total solids content, the solution was cooled to 20°C and spray-dried. The solubility of lactose at 20°C is 15 g/100 g water.

Using the variation of solubility with temperature, we produced lactose powders with varying degrees of crystallinity as follows: a 25% solution of lactose, prepared at 50°C, was divided into 3 portions. Each portion was brought to a different temperature (20, 40 or 60°C) before drying. The proportion of amorphous lactose in the spray-dried powder was higher for higher pre-drying solution temperatures than for samples fed to the dryer at lower temperature (Table 4). At 20°C a 25% solution is supersaturated. Possibly, microcrystals were formed in the feed solution or in the droplets shortly after atomization, resulting in a more crystalline powder at the end of the process. We did not, however, observe directly the presence of crystals in the feed solution.

Completely amorphous lactose powder was obtained from a 15% solution, brought to 50°C before spray-drying. The powder thus obtained was then completely dehydrated over P$_2$O$_5$. Powders of the various milk components (Table 1) were obtained by spray-drying solutions containing 20% dry matter (total solids).

Diacetyl retention

Due to its intermediate volatility, diacetyl (2,3-butanedione) is a convenient model for studying retention of volatiles in spray-drying. Diacetyl content of the feed solutions and the powders was determined by a colorimetric method, adapted from AOAC (1990) method 978.11, after recovery by distillation. Retention during spray-drying was defined as:

Table 1—Composition of raw materials

Product (powder)[a]	Composition % w/w
lactose	α-lactose monohydrate with 2% of β-lactose
skim milk (1)	water content = 5% maxi fat = 1.5% maxi protein = 35% maxi lactose = 52%
whole milk (2)	fat = 26% maxi protein = 25.5% maxi lactose = 38.5% ash = 6.3–6.5%
ultrafiltration retentate = proteins + inorganic substances (3)	water content = 4.5% total nitrogenous substances = 79.3% non protein nitrogen = 0.5% inorganic substances = 7.8%
whey permeate = lactose + inorganic substances (3)	water content = 2.1% lactose = 77% total nitrogenous substances = 4.3% non protein nitrogen = 0.3% inorganic substances = 9.7%
anhydrous fat (4)	

[a] (1) Sigma; (2) Prolait, Niort (F); (3) INRA, Rennes (F); (4) Dairy Fléchard, Bagnoles de l'Orne (F).

$$\frac{\text{diacetyl content of the powder (mg/kg dry matter)}}{\text{diacetyl content of the feed solution (mg/kg dry matter)}}$$

Retention in powders during storage was expressed as percentage of initial concentration.

Differential scanning calorimetry (DSC)

The instrument used was a Mettler TA 4000 Thermal Analysis System with TC 11 TA processor and TA 72 PS.2 software, calibrated for temperature and flux with indium. Thermograms were used to determine glass transition temperature T_g, crystallization temperature T_{cr} and heat of crystallization ΔH_{cr} (Fig. 1). All measurements were performed at 5°C/min between −20°C and 250°C depending on the location of T_g and T_{cr}, using sealed aluminum pans (Mettler 40 μL) and an empty pan as a reference. ΔH_{cr} was calculated by integration of the crystallization peak (Roos and Karel, 1992).

Evaluation of amorphous fraction in lactose powders

The degree of crystallinity of lactose powders was calculated from their ΔH_{cr}. The heat of crystallization of amorphous lactose at 0% humidity was taken as $\Delta H_{cro} = 97$ J/g (Fig. 1). The heat of crystallization of partially crystallized lactose, ΔH_{cr}, is proportional to the percentage of amorphous lactose in the sample. Assuming ΔH_{cro} independent of water content (Roos and Karel, 1992):

% amorphous fraction in lactose powder = $\Delta H_{cr}/\Delta H_{cro}$

For calculation of the amorphous lactose content in milk powder, the total lactose content of the milk powder, X g/g, must be included in the calculation, thus:

% amorphous lactose in milk powder = $\Delta H_{cr}/X \cdot \Delta H_{cro}$

Storage of powders at different relative humidities

The spray-dried lactose and skim milk powders were stored in closed glass containers (1L), in which a constant relative humidity was maintained through the presence of saturated salt solutions. The following salt solutions were used: potassium acetate for 22% RH, magnesium chloride for 32% RH, potassium carbonate for 43% RH and magnesium nitrate for 53% RH. The jars were kept at 25°C. The extents of crystallization and diacetyl retention were determined at intervals of 48 hr storage time. Each glass jar was opened only once to supply one sample. Equilibrium a_w was reached in about 24 hr and the changes in the powders was observed during 30 days.

Storage of powders at different T − T_g values

The spray-dried lactose and skim milk powders were first equilibrated in jars at different temperature and different relative humidities. Constant

Table 2—Storage conditions for lactose and skim milk powders with different $T-T_g$

	Relative humidity (%)	Lactose T_g (°C)	Storage temp (°C) (relative humidity %)	$T-T_g$ (°C)
Lactose powder	53 (2°C)	2	5 (53%)	3
	43 (2°C)	19	30 (43%)	11
	32 (25°C)	39.5	45 (32%)	5.5
	32 (25°C)	39.5	52 (32%)	12.5
Skim milk powder	22 (25°C)	58	75 (22%)	17
	32 (25°C)	39	50 (32%)	11
	43 (2°C)	19	25 (43%)	6

Rate 5.0 °C/min

crystallization

Glass transition
Onset 94.6 °C
Midpt 97.7 °C
Inflpt 98.4 °C
Endpt 100.8 °C
Δcp 0.38 j/g.K

Integration
ΔH 1595 mJ
96.1 J/g
Peak T_{cr} 154.4 °C
50.8 mW
Ref ΔH_{cro} 97.0 J/g
Content 99.0 %

melting

Tg
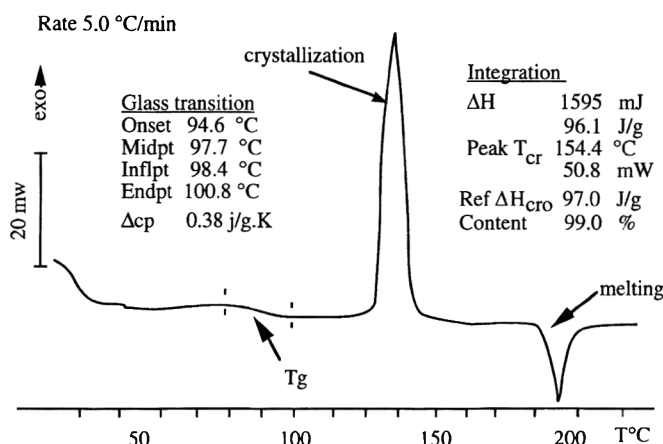
Fig. 1—Thermogram of amorphous lactose powder determination of T_g, T_{cr}, ΔH_{cr}.

T_g was reached after 24 hr. The samples were then maintained at a constant temperature higher than their T_g, in order to obtain different values of $T - T_g$ for the different relative humidity levels (Table 2).

RESULTS & DISCUSSION

Retention of diacetyl during spray-drying

Lactose solutions. Loss of diacetyl during spray-drying decreased as concentration of lactose in the feed solution increased (Table 3). This was in accordance with other reports on aroma retention in spray-drying (Reineccius and Bangs, 1985; King, 1994). The data (Table 4) indicate a strong relationship between diacetyl retention and the amorphous/crystalline ratio. Retention was higher when the drying conditions led to a powder with a higher proportion of crystalline lactose. This result may be explained as follows. The loss of volatile aroma substances in spray-drying is largely governed by the effective diffusivity of a volatile compound across the droplet. As water evaporates and the solutes in the droplet reach a certain concentration, microcrystals are formed within the supersaturated solution. Since spray-drying is a very fast process, the physical composition of the drop is rapidly fixed and reflected in the structure of the resulting powder particle. Thus, the resulting powder may be completely amorphous or it may contain microcrystalline regions dispersed in the amorphous mass. In the droplet the microcrystals constitute an additional barrier to diffusion of volatile molecules. Their resistance to mass transfer becomes more important when they are present in the droplet in greater proportion, thus increasing retention of volatiles in the droplet during drying. The future observation of the structure of droplets during drying might help elucidate this.

Effect of proteins and inorganic substances. Retention data for skim milk and different fractions of milk were compared (Table 3). When dried under the same conditions, milk proteins (UF retentate) show a higher retention capacity (62%) than lactose (41%). The higher retention capacity (53%) of whey permeate (lactose + minerals) compared to lactose may indicate a

Table 3—Retention of diacetyl in lactose and milk products during Leaflash spray-drying (diacetyl 0.5% of total solids (TS); drying air temperatures 250/100°C)

Sample		ns (g water/100g TS)	Retention (%)	Total solids content (%)
Lactose	1	3.33 ± 0.02	58.0 ± 0.5	30
	2	3.02	60.3	30
	3	2.96	61.1	30
	4	2.71	58.5	30
	5	2.83	57.2	30
mean value		2.97	59.0	30
Lactose		4.30 – 4.32	40.7 – 42.6	20
Ultrafiltration retentate (protein)		6.58 – 6.39	61.5 – 62.9	20
Whey permeate (lactose + salts)		3.98 – 3.90	52.1 – 53.9	20
Whey permeate + lactose (10% + 10%)		3.72 – 3.86	45.2 – 47.0	20
Ultrafiltration retentate + whey permeate		5.60 – 5.49	56.0 – 58.9	20
Skim milk		5.76 – 5.87	60.9 – 61.1	20

Table 4—Retention of diacetyl during Leaflash spray-drying of aqueous lactose solutions in relation with % of amorphous lactose in powder (Total solids, TS = 25%; drying air temperatures 250/100°C)

		Sample 1	Sample 2	Sample 3
Temperature of solution	(°C)	60	40	20
ns	(g water/100g TS)	3.19	3.48	2.68
T_g	(°C)	70.1	64.4	69.5
T_{cr}	(°C)	123.1	116.3	120.2
ΔH_{cr}	(J/g)	75.7	67.4	54.8
Amorphous lactose	(%)	78.0	69.5	56.5
Retention	(%)	39.2	42.9	51.7

Table 5—Retention of diacetyl in relation to fat content

Product	Composition (g/100g total solids)			Total solids content (%)	Retention (%)
	lactose	protein	fat		
Skim milk	52	35	1	20	65.6
3/4 Skim milk + 1/4 Whole milk	48.4	32.5	7	20	64.2
1/2 Skim milk + 1/2 Whole milk	45	30.3	13.5	20	61.8
1/4 Skim milk + 3/4 Whole milk	41.1	27.6	21	20	62.1
Whole milk	38.5	25.9	26	20	59.6

	Composition (g/kg solution)			Total solids content (%)	Retention (%)
	lactose	protein	fat		
Ultrafiltration retentate	0	200	0	20	62.0
Ultrafiltration retentate + 10% fat	0	180	20	20	58.1
Ultrafiltration retentate + 10% fat	0	200	20	22	65.1

Fig. 2—T_g and T_{cr} of lactose in relation to a_w (storage at constant relative humidity).
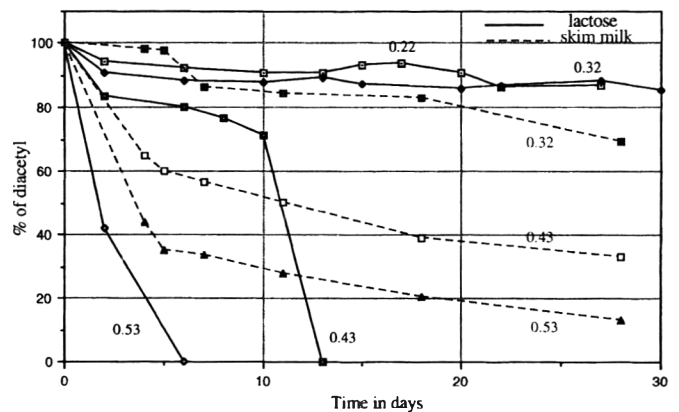
Fig. 3—Loss of diacetyl in powder during storage in relation to time and relative humidity at 25°C.

Fig. 4—Rate of crystallization of lactose during storage in relation to time and relative humidity at 25°C.

positive contribution of inorganic constituents. This was confirmed by the intermediate retention (46%) in the whey permeate + lactose sample. We do not have an explanation for the observed positive effect of inorganic constituents on diacetyl retention. The mixture of UF retentate of skim milk and UF permeate of whey had a composition close to that of skim milk. Its diacetyl retention capacity was lower than that of the UF retentate alone and almost equaled that of skim milk.

Effect of milk fat. Diacetyl retention data for milk and milk derivatives with different levels of fat content were compared (Table 5). Retention seemed to be impaired as fat content increased. This may be due to the fact that, at constant dry matter concentration in the feed solution, any increase in fat content

would include a decrease in protein and lactose, the main systems responsible for volatiles retention. This explanation is supported by the retention data for UF retentate with or without fat. At constant dry matter content, addition of fat (with reduction of protein) reduces retention. Compensation of the replacement by increasing the total dry matter content from 20 to 22% restored the higher retention values.

Retention of diacetyl during storage of the powders

Lactose crystallization and diacetyl retention. DSC measurements on amorphous lactose powders after equilibration at
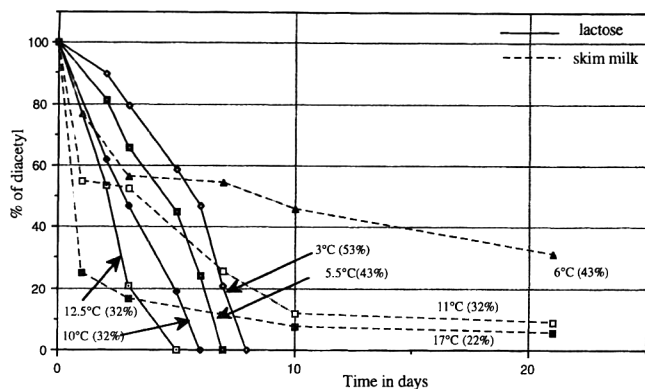
Fig. 5—Loss of diacetyl in powder during storage in relation to time and T - T_g.
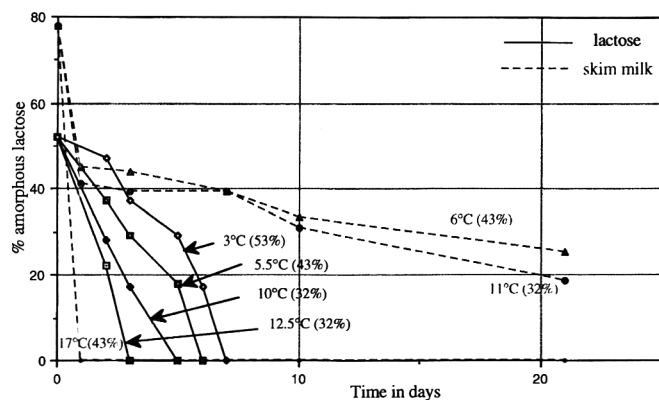
Fig. 6—Rate of crystallization of lactose during storage in relation to time and T - T_g.
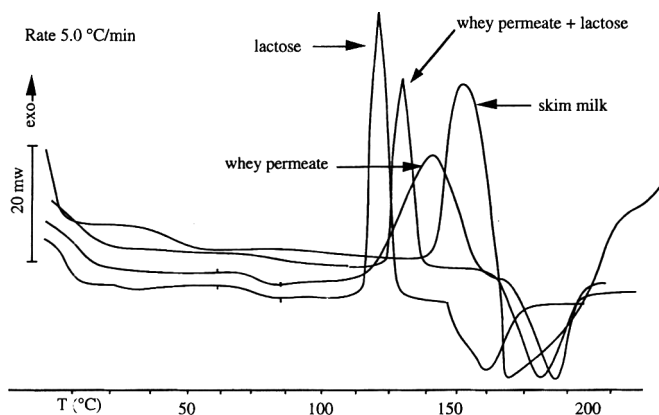
Fig. 7—Thermograms DSC of milk components.

different levels of a_w showed a linear relationship between a_w and the temperatures of crystallization (T_{cr}) and glass transition (T_g) within the a_w range 0 to 0.5 (Fig. 2). The results were comparable to those of Roos and Karel (1991a, b). The difference between T_g and T_{cr} was 52 ± 2°C.

In lactose and skim milk powders, a close relationship between diacetyl loss and crystallization of lactose was observed. Volatile loss follows crystallization after a lag time longer for skim milk than for lactose (Fig. 3 and 4). Crystallization was faster and extent of diacetyl loss was larger at higher a_w. In lactose at a_w of 0.32 and 0.22, where T_g (39 and 52°C, respectively) was higher than the storage temperature (25°C), crystallization was very slow and diacetyl was almost totally retained. In contrast, at a_w of 0.53, where T_g was more than 20°C lower than the storage temperature, crystallization was almost instan-

taneous and practically all diacetyl was lost after 6 days. At the intermediate a_w (0.43) crystallization was slower at first and very rapid afterwards. The rate of diacetyl loss followed the same pattern. In skim milk, even after complete crystallization of lactose (a_w = 0.53), part of the diacetyl was still retained, probably adsorbed by other components, mainly proteins.

Changes in stored powders as a function of T − T_g. Examination of extent of crystallization and diacetyl loss data in lactose powder stored at different temperatures and a_w values (corresponding to different values of T_g) showed that both rate of crystallization and diacetyl loss increased with increasing T − T_g (Fig. 5, 6). The same relation was seen for lactose in skim milk powder but the rate of crystallization was slower in skim milk. Examination of the corresponding DSC thermograms (Fig. 7) showed for skim milk, a crystallization peak which was low and wide, indicating slower crystallization. The crystallization temperature T_{cr} for skim milk is higher than for pure lactose for the same a_w. The T_{cr} − T_g difference (about 50°C for lactose) becomes 70°C for whey permeate and milk. This observation seemed to confirm the retarding effect of non-lactose components on lactose crystallization.

CONCLUSION

AMONG THE COMPONENTS OF MILK, the proteins seemed to have the strongest positive effect on retention of diacetyl during spray-drying. Conditions which promote formation of microcrystals in the powder also enhanced diacetyl retention. During spray-drying, diacetyl is probably retained through a mechanism of occlusion in an amorphous matrix containing microcrystals. During storage of lactose or skim milk powder, diacetyl loss closely followed the crystallization of lactose. Therefore, the retention of volatile aroma compounds could be improved by factors which retard lactose crystallization such as low relative humidity, low temperature, and the presence of proteins and inorganic substances.

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Sensory Evaluation of Reduced Fat Cheeses

M.A. DRAKE, W. HERRETT, T.D. BOYLSTON, and B.G. SWANSON

──────── ABSTRACT ────────

The objectives of this research were to compare the effects of seven make-procedures on the sensory quality of reduced fat Monterey Jack-type cheeses. Modifications (decreased cook temperature, decreased ripe time, decreased starter, homogenization, added milk-solids-not-fat, added flavor cultures, and washed curd) were incorporated into standard make-procedures for 33% reduced fat (RF) Monterey Jack-type cheeses. Two controls, a standard full fat cheese, and a standard RF cheese, were also prepared. Cheeses were aged 3 and 7 mo prior to sensory evaluation. The full fat control cheese and the washed curd RF cheese received the highest flavor scores from trained dairy judges (n = 9) and the highest flavor and acceptance scores from a consumer panel (n = 125). Washed curd was an effective modification for optimum sensory quality of RF cheeses.

Key Words: reduced fat, cheese, sensory evaluation, acceptability

INTRODUCTION

REDUCED FAT (RF) DAIRY PRODUCTS are the most widely consumed RF foods (Thayer, 1992). RF cheeses present a challenging problem because fat is important to texture and flavor (Jameson, 1990; Olson, 1990; Rosenberg, 1992). Fat reduction in hard and semi-hard cheeses results in undesirable rubbery texture, lack of flavor, and/or presence of off-flavors (Olson, 1990). A make-procedure is a standard list of procedures or "recipe" that is followed to make a specific type of cheese. Many make-procedure variations have been suggested to maximize sensory quality of RF cheeses (Deane and Dolan, 1975; Banks et al., 1989; McGregor and White, 1990; Olson, 1990; Chen, 1991; Banks et al., 1993; Metzger and Mistry, 1993), but few have been directly compared to evaluate effectiveness. Trained sensory panels evaluated many RF cheeses prepared with make-procedure modifications, but exhaustive sensory evaluation using trained, difference, and acceptance panels has not been reported.

The Washington State University Creamery markets a cheese (Viking) that is similar in composition to Monterey Jack. The cheese is generally marketed between 3 and 6 mo of age. A RF version of this cheese similar in quality to its full-fat counterpart is desired. The objectives of our research were to compare the effects of seven different make-procedures on sensory quality of RF Monterey Jack type cheeses.

MATERIALS & METHODS

Cheese manufacture

Whole milk was obtained from the Washington State University dairy farm (Pullman, WA) and skim milk was purchased from Darigold, Inc. (Spokane, WA). Lactic cultures (mixed strains of *Lactococcus lactis* ssp *lactis* and *Lactococcus lactis* ssp *cremoris* were purchased as frozen rediset cultures (CHR. Hansen, Milwaukee, WI). Lactic cultures were then grown in Phase 4 media according to manufacturer's directions (Marschall Products, Madison, WI) prior to use in cheese. *Streptococcus salivarius* ssp *thermophilus* (CHR Hansen, Milwaukee, WI) and *Lactobacillus helveticus* (WSU isolate) were cultured in sterilized whole milk. Whole milk (136 kg) was pasteurized at 63°C for 30 min and made into Viking cheese (Fig. 1). This cheese served as the full fat (FF) control. For the RF cheese treatments, whole milk was pasteurized at 63°C

for 30 min prior to standardization to 2.5% fat with skim milk to make a total weight of 136 kg. Seven make-procedure modifications plus a RF control were made (Table 1). Duplicate samples were taken prior to packaging for proximate analysis. The cheeses were vacuum sealed under 58.4 cm mercury in 603 by 204 cans and aged at 7.2°C until sensory evaluation. Composition of cheeses is given in Table 2, and changes in pH during aging at 7.2°C in Table 3.

Proximate analysis

Protein content of the milk was determined by a UDY dye binding colorimeter protein analyzer (UDY Corp., Fort Collins, CO) according to standard methods of analysis (Bradley et al., 1993). Fat content of standardized milks was determined by the Babcock method (Bradley et al., 1993). Fat, salt, moisture and pH of the cheeses were determined according to standard methods of analysis (Bradley et al., 1993).

Sensory evaluation

Cheeses were evaluated by a trained dairy products evaluation panel (n = 9), a prescreened difference panel (n = 30), and an acceptance

┌───┐
│ Pasteurize milk at 63°C for 30 min   │
│ Cool to 31°C   │
│ Add starter[a] and ripen for 50 min   │
│   │
│ Add rennet at the rate of 10 mL / 44kg   │
│ Set for 30 min   │
│   │
│ Cut curd and allow to heal for 5 min   │
│   │
│ Cook by raising the temperature to 38°C   │
│ over a 50 min time period   │
│   │
│ Drain the whey and shape the curd into   │
│ loaves. Cheddar the loaves for 45 min   │
│   │
│ Mill and salt at the rate of 0.32% of   │
│ milk weight   │
│   │
│ Hoop and press for 2.5 h   │
│ Package   │
└───┘

Fig. 1—Make-procedure for Viking cheese. Procedure originally adapted from Czulak and Hammond (1958). [a]Cultures added include a lactic culture (0.35% w/w), *Lactobacillus delbrueckii* ssp *bulgaricus* (0.11% w/w), and *Streptococcus salivarius* ssp *thermophilus* (1.0% w/w). Cultures were purchased from Chr. Hansen (Milwaukee, WI).

Table 1—Reduced-fat (RF) make-procedure modifications

Treatment	Modification
Standard RF Viking	0.30% lactic culture, cook to 36.5°C[a]
Washed-curd (WC)	Drain half of the whey (v/v) at dipping and add 35°C water for 5 min
Milk-solids-not-fat (MSNF)	Add 1.2% (w/w) MSNF prior to pasteurization
Decreased starter (DS)	0.22% lactic starter
Low cook temperature (LC)	Cook to 35°C
Homogenization (H)	Homogenize whole milk post-pasteurization 6900 kPa
Flavor culture (FC)	0.1% (w/w) CR210[b] and 0.045% (v/w) 15× starter distillate[c]
Decreased ripe time (DR)	decrease ripe time to 30 min

[a] All of the RF treatments contain 0.30% lactic culture and a final cook temperature of 36.5°C.

[b] CR210 is a lactose-negative proteolytic lactic adjunct starter for RF cheeses purchased from CHR.Hansen (Milwaukee, WI).

[c] 15× starter distillate purchased from CHR. Hansen (Milwaukee, WI).

Table 2—Composition of cheeses

Treatment	Mean[a] %Fat	Mean[a] %Salt	Mean[a] %Moisture	Salt/Moisture
FF Viking	33.33[a] (0.29)	1.58[c] (0.02)	40.53[g] (0.31)	3.90
RF Viking	22.33[b] (0.29)	1.44[e] (0.01)	47.13[cd] (0.28)	3.06
MSNF	21.00[d] (0.29)	1.39[f] (0.03)	47.40[bc] (0.11)	2.93
DS	22.33[b] (0.50)	1.40[ef] (0.02)	46.80[ef] (0.12)	3.00
LC	21.17[cd] (0.29)	1.53[d] (0.03)	49.33[a] (0.23)	3.10
H	21.33[cd] (0.50)	1.63[b] (0.01)	47.40[bc] (0.20)	3.45
DR	21.17[cd] (0.29)	1.67[a] (0.03)	47.73[b] (0.12)	3.50
WC	21.00[d] (0.29)	1.67[a] (0.02)	46.53[f] (0.12)	3.59
FC	21.67[c] (0.29)	1.61[bc] (0.01)	47.07[cd] (0.20)	3.42

[a] Means are the result of three replicates. Fat and salt contents are calculated on a wet weight basis. Means in a column followed by the same letter are not significantly different (P≤0.05). Numbers in parentheses are the standard deviation of the means.

Table 3—Changes in pH during aging at 7.2°C

Treatment	Time 0[a]	3 mo	7 mo
FF Viking	5.23	5.28	5.30
RF Viking	5.13	5.10	5.04
MSNF	5.23	5.15	5.03
DS	5.29	5.18	4.99
LC	5.13	5.10	5.04
H	5.31	5.00	4.71
DR	5.43	5.19	4.86
WC	5.31	5.43	5.16
FC	5.24	5.16	4.98

[a] pH taken within 1 week after make.

panel (n = 125) after 3 and 7 mo aging. All panelists signed a Washington State University human subjects approval form. Panelists had unconstrained access to deionized water, unsalted soda crackers, and green seedless grapes during testing to help cleanse their palates.

Judges trained in evaluating dairy products were chosen from WSU Creamery employees that previously completed a class in dairy products evaluation and were familiar with the standard American Dairy Science Association (ADSA) scoring system (Bodyfelt et al., 1988). Prior to the 3 mo sensory evaluation, judges participated in a 3 hr "review" session of common cheese defects given by the instructor of the dairy products evaluation class. Cheeses were assigned random two-digit numbers and judges evaluated cheeses in a randomized balanced block design. Each cheese was evaluated using the standard ADSA scoring sheet. The evaluation was carried out under white lights.

For the difference panel, untrained individuals were prescreened for ability to detect differences in fat content, texture, and flavor of cheeses. Volunteers (63) participated in the screening panel. Panelists were asked to choose the different cheese in a series of three standard triangle tests. Red lights were used in tasting booths to mask color differences. The

cheeses were presented in a randomized balanced block design. The first test screened potential panelists for ability to detect texture differences and included a regular Viking cheese and a commercial high fat Havarti cheese. The second test consisted of a regular Viking cheese and a RF Viking cheese. The third test consisted of a commercial mild Cheddar cheese, aged 3 mo, and a commercial extra sharp Cheddar cheese aged 12 mo. Panelists who scored the three triangle tests correctly (32) were asked to participate on the taste panels to evaluate the RF Viking cheeses. Selected sensory panelists participated in a total of 29 triangle tests on 9 separate days when the cheeses were 3 and 7 mo of age.

Faculty, staff, and students university-wide were solicited for acceptance panels. Tasting was carried out under white lights. Prior to tasting, panelists completed a questionnaire asking frequency of cheese consumption (<1 once per wk, 2–3 times per wk, 4–5 times per wk, or >5 times per wk) and cheese preference (processed, mild to medium, or sharp). To eliminate confusion about types of cheese, examples of the cheese types were given (eg. processed (Velveeta, processed American slices, Cheese Whiz)). For acceptance rating, cheeses were assigned a three-digit random number and introduced one sample at a time. Panelists were asked to evaluate their perception of firmness, texture, flavor and overall acceptance of each sample on a 9-point hedonic scale. Panelists were instructed to clear their palates before proceeding to the next sample. Due to the large number of treatments, it was not feasible to evaluate all cheeses on one day. To avoid panelist fatigue, 2 days of acceptance testing were conducted. Cheeses were presented in a 2-day randomized unbalanced incomplete block design with a split plot over time. Each day the FF control and the RF control were evaluated vs 2 or 3 of the experimental RF treatments. Since the RF and the FF control were the same sample on both days and due to the large number of acceptance panelists on each day (n = 125 each day), scores for these control cheeses were expected to not be significantly different from day to day. Treatments could then be compared to control cheeses and to treatments evaluated on different days provided the reference control samples from both days were not significantly different.

Statistical analysis

Significance was established at $P \leq 0.05$. Significant differences between means were determined using least square means. An analysis of variance (ANOVA) was performed on fat, salt, and moisture content of the cheeses. Significance of triangle tests was determined using the expanded statistical tables of Roessler et al. (1978). Trained dairy judging results were analyzed with an ANOVA and significance between means determined. Dairy judge data were also sorted by panelist to determine if individual panelists scored the cheeses differently. Acceptance data for both days were combined after determining that the FF and RF control cheese scores, respectively, for both days were not significantly different. An analysis of variance was run to determine significant treatment effects. Acceptance data were also sorted and analyzed by preferred cheese-type and frequency of cheese consumption to determine if these variables influenced perception of firmness, texture, flavor and acceptance.

RESULTS & DISCUSSION

Composition

Percent fat and protein of whole milk used for the FF control was 3.80 and 3.16, respectively. Milk-solids-not-fat (MSNF) supplemented milk contained 3.55% protein. Mean percent fat and protein contents of reduced-fat milks were 2.50 ± 0.05 and 3.17 ± 0.06, respectively. Proximate analysis results of cheeses are presented in Tables 2 and 3.

Trained and difference panels

Results of trained dairy judges are listed in Tables 4 and 5. No significant panelist effect was detected at 3 or 7 months. At the 3 mo evaluation, screened difference panelists detected significant differences ($P \leq 0.05$) between each of the RF cheeses and the FF control cheese. Difference panelists also detected significant differences ($P \leq 0.05$) between the RF control and each RF treatment except the decreased starter (DS) treatment. At the 7 mo evaluation, panelists did not detect significant differences between the RF control cheese and the DS, decreased ripe time (DR), or decreased cook temperature (DC) RF cheeses. Other RF cheeses were determined to be significantly different

Table 4—Trained dairy judging results after three months aging

Treatment	Mean[a] flavor	Mean[a] texture	Defects[b] Flavor	Texture
FF Viking	8.5[a] (0.76)	3.75[a] (0.46)	bitter, high acid	open
RF Viking	7.75[a] (1.11)	3.13[b] (0.83)	bitter, high acid	open, weak
MSNF	8.13[a] (1.00)	3.63[ab] (0.52)	bitter, high acid	open
DS	7.75[a] (0.88)	3.50[ab] (0.53)	bitter, high acid	open, weak
LC	6.50[b] (1.20)	3.88[a] (0.31)	bitter, high acid whey taint	open
H	6.75[b] (0.88)	2.25[c] (0.71)	bitter, high acid whey taint	open, crumbly mealy
DR	7.75[a] (0.71)	3.13[b] (0.64)	flat, no flavor whey taint	open, weak
WC	8.50[a] (0.53)	3.88[a] (0.35)	flat, no flavor bitter	weak
FC	8.0[a] (1.02)	3.13[b] (0.64)	bitter, high acid	open, gassy

[a] Means are the calculated averages of scores from nine judges.

[b] Defects are listed in the ADSA score sheet. Only defects noted by five or more judges are listed. Means in a column followed by the same letter are not significantly different (P ≤ 0.05). Numbers in parentheses are the standard deviations of the means. For flavor, 10 = superior quality, 1 = lowest quality. For texture, 5 = highest quality, 1 = lowest quality.

Table 5—Trained dairy judging results after seven months aging

Treatment	Mean[a] flavor	Mean[a] texture	Defects[b] Flavor	Texture
FF Viking	7.77[a] (0.89)	3.33[ab] (0.60)	bitter	open, weak
RF Viking	5.78[bc] (1.09)	3.22[abc] (0.67)	bitter, high acid	open
MSNF	6.44[b] (0.88)	2.67[c] (0.87)	bitter, high acid	open, weak
DS	5.78[bc] (1.20)	3.33[ab] (0.71)	high acid, rancid	open
LC	5.00[c] (1.11)	2.89[b] (0.93)	bitter, high acid rancid	open, weak
H	3.78[d] (0.83)	1.67[d] (0.50)	bitter, high acid whey taint	open, short crumbly
DR	5.78[bc] (0.97)	3.11[abc] (0.60)	bitter, high acid	open
WC	7.11[ab] (0.83)	3.56[a] (0.53)	bitter	weak
FC	6.44[b] (0.93)	3.44[ab] (0.53)	bitter, high acid	open

[a] Means are the calculated averages of scores from nine judges.

[b] Defects are listed in the ADSA score sheet. Only defects noted by five or more judges are listed. Means in a column followed by the same letter are not significantly different (P ≤ 0.05). Numbers in parentheses are the standard deviations of the means. For flavor, 10 = superior quality, 1 = lowest quality. For texture, 5 = highest quality, 1 = lowest quality.

from the RF control cheese. Significant differences were not detected between the washed curd (WC) RF cheese and the FF control cheese. The other RF cheeses were significantly different from the FF control cheese.

Acceptance panels

Results of the acceptance panels (Tables 6 and 7) showed flavor scores and overall acceptance of the FF and RF control cheeses were not significantly different from day to day at 3 or 7 mo (P ≤ 0.05). At the 3 mo evaluation, firmness and texture scores for the RF and FF control cheeses were different, indicating that panelists had difficulty perceiving these attributes among the cheeses or that differences existed in panelist interpretation of the terms. Treatment means for these attributes were not compared and data are not given. The DS cheese was not included in the 3 mo sensory acceptance evaluation since this cheese was not different from the RF control based on difference tests. At the 7 mo acceptance evaluation, the DS, DR, and DC RF cheeses were not different from the RF control cheese according to the difference panelists and were not included in acceptance evaluations. Neither panelist cheese preference nor

Table 6—Consumer panel results after three months aging

Treatment	Mean[a] flavor	Mean[a] overall acceptance
FF Viking	5.74[a] (1.01)	6.57[a] (1.10)
RF Viking	5.60[a] (1.18)	5.60[c] (1.04)
MSNF	5.86[a] (1.53)	5.95[b] (1.48)
LC	5.52[a] (1.53)	5.03[d] (1.39)
H	5.03[b] (1.67)	4.42[e] (1.51)
DR	4.74[b] (1.54)	5.47[c] (1.49)
WC	5.69[a] (1.52)	6.47[a] (1.38)
FC	5.59[a] (1.41)	5.61[bc] (1.41)

[a] Means of the RF and FF controls are calculated from scores on two days of acceptance testing (n = 250). Other means are the calculated averages of 125 scores. Means in a column followed by the same letter are not significantly different (P ≤ 0.05). Numbers in parentheses are the standard deviation of the means. For flavor, 1 = low or lack of flavor. 9 = high amount of flavor. For acceptance, 1 = dislike extremely. 9 = like extremely.

Table 7—Consumer panel results after seven months aging

Treatment	Mean[a] firmness	Mean[a] texture	Mean[a] flavor	Mean[a] overall acceptance
FF Viking	4.46[d] (1.46)	4.94[d] (1.47)	6.33[a] (1.38)	6.74[a] (1.10)
RF Viking	5.03[bc] (1.51)	4.55[c] (1.40)	5.80[bc] (1.21)	5.54[b] (1.10)
MSNF	5.39[b] (1.47)	5.17[b] (1.51)	5.71[c] (1.58)	5.62[b] (1.36)
H	6.46[a] (1.35)	6.55[a] (1.21)	5.10[d] (1.61)	3.90[c] (1.31)
WC	4.18[d] (1.49)	3.73[d] (1.27)	5.95[b] (1.25)	6.53[a] (1.15)
FC	5.00[c] (1.49)	4.74[bc] (1.44)	5.45[c] (1.41)	5.58[b] (1.37)

[a] Means of the RF and FF controls are calculated from scores on both days of acceptance testing (n = 250). Other means are the calculated averages of 125 scores. Means in a column followed by the same letter are not significantly different (P ≤ 0.05). Numbers in parentheses are the standard deviation of the means. For firmness, 1 = softest, 9 = firmest. For texture, 1 = creamy, 9 = crumbly. For flavor, 1 = low or lack of flavor, 9 = high amount of flavor. For acceptance, 1 = dislike extremely, 9 = like extremely.

frequency of cheese consumption affected the data. Of the acceptance panelists surveyed on both days at 3 and 7 mo (n = 500), 95% consumed cheese at least two to three times a week.

Differences in proximate analysis data were attributed to differences in make-procedure since the milk was standardized to 2.5% fat and 0.32 % (w/w) salt was added to each batch. The WC treatment was the best rated RF cheese at 3 and 7 mo and was the only RF cheese that exhibited an initial increase in pH. Often, pH of RF cheeses continues to decrease rather than increase during aging. Due to lower salt to moisture ratios in RF cheeses, culture activity may not be arrested (Thunell, 1992). A pH decrease during aging results in a high acid flavor that continues to deteriorate with further aging. WC also contained one of the highest salt contents and the highest salt/moisture ratio. The DR cheese contained the highest salt content and a high salt/moisture ratio but received much lower trained panel flavor scores at 3 and 7 mo and was not different from the RF control cheese at 7 mo.

Washing the curd is common with Monterey Jack and Colby cheeses (Kosikowski, 1977). Deane and Dolan (1975) reported an increase in sensory quality of washed curd RF cheeses over unwashed RF cheeses. In RF cheeses, curd-washing can wash away acid and lactose and prevent an initial pH drop, but can also wash away desirable flavor compounds. Washing the curd of RF cheeses is reported to contribute to meaty brothy flavors (Thunell, 1992). Chen (1991) and Chen and Johnson (1993) reported that RF cheeses prepared without washing the curd were preferred over RF washed curd cheeses and that there was a lack of flavor in young washed curd cheeses at 1.5 mo. and meaty off-flavors in older cheeses at 3 mo. Chen and Johnson (1993) used longer washing times such as diluting the whey prior to cooking and 20 min "soaks" after milling.

In our study, the wash procedure was more of a post-cook rinse; half of the whey was drained at the normal dipping time,

35°C water added back to original whey volume and the curd rinsed for 5 min. Dipping, cheddaring and milling then proceeded normally. As noted by Chen (1991), washing or rinsing curd at a temperature similar to the actual curd temperature did not affect moisture content of the cheese. The washed curd cheese in our study did not have increased moisture content over the other RF cheeses. A lack of flavor in the washed curd (WC) cheese was noted by trained dairy judges at 3 mo, but a lack of flavor was not reported at 7 mo. Combining a WC make-procedure modification with other modifications such as decreased ripe time and/or an adjunct culture to accelerate flavor development may further improve this cheese. The WC cheese received higher texture scores than the FF control cheese. The WC cheese exhibited a smooth waxy texture. WC cheese scored higher overall scores at 3 and 7 mo than the RF control cheese which involved no washing of the curd. Overall acceptance scores of the WC cheese were not different from the FF control. Interestingly, the WC cheese was also perceived by acceptance panels to be softer and creamier than the FF control cheese after 7 mo.

A rancid flavor was detected in the low cook temperature (LC) treatment by the dairy judges at 7 mo. The difference panelists did not detect differences between the LC cheese and the RF control at 7 mo. The lack of a difference from the RF control may be due to difficulty in cleaning a rancid flavor from the palate. Many difference panelists noted that some of the cheeses aged for 7 mo were very difficult to clean from the mouth. Decreasing the cook temperature of cheese increases the amount of moisture retained by the curd (Olson, 1990). As expected, the LC cheeses exhibited a significantly larger moisture content and a lower salt/moisture ratio from the other RF treatments. The salt/moisture ratio is an indicator of the true salt content in the moisture phase of the cheese. Low salt/moisture ratios can result in proliferation of nonstarter bacteria, acceleration of enzymatic reactions, and altered metabolism of normal starter bacteria (Chen, 1991). The rancid flavor of the LC cheese may be a result of high moisture and low salt/moisture ratio.

Metzger and Mistry (1993) reported that homogenization of cream at 13800 kPa (10300 kPa first stage, 3400 kPa second stage) prior to standardization with skim milk improved the texture of RF Cheddar cheeses aged 3 mo and did not have an effect on flavor or appearance of the cheeses. The homogenized milk (H) RF cheese in our study was prepared similarly to Metzger and Mistry (1993); whole milk was homogenized prior to standardization with skim. The H RF cheese was visibly more opaque and a brighter white color than the other cheeses. Viking cheese does not have added coloring and normally has a pale creamy yellow color. H RF cheese received lower texture scores from the dairy judges at 3 and 7 mo and scored the lowest overall acceptance scores at 3 and 7 mo. The H cheese was also firmer and more crumbly than the other cheeses according to the acceptance panel at 7 mo. These firmness and texture acceptance scores agreed with remarks by trained panelists at 3 and 7 mo that the H RF cheese was crumbly, but the other cheeses were not. The H RF cheese also had a tendency to whey-off. Free whey was present in the H RF cheese containers at 7 mo.

The addition of MSNF to RF milk prior to cheesemaking is recommended to increase total solids and buffering capacity (Olson, 1990). Anderson et al. (1993) also reported that the use of condensed milk with a higher total solids content to manufacture reduced fat Cheddar cheese improved flavor development. The MSNF cheese in our study exhibited the lowest salt content and the lowest salt/moisture ratio, but the pH did not decrease as sharply as some of the other treatments. This observation may have been due to an increased buffering capacity. However, the MSNF RF treatment was perceived to have a high acid and bitter flavor by dairy judges at 3 and 7 mo. Anderson et al. (1993) reported that reduced fat cheeses manufactured with condensed milk were firmer and more crumbly. The MSNF cheese also received high firmness and crumbliness scores from the acceptance panel, as expected in a high milk solids cheese.

Adjunct starter cultures are recommended for increasing desirable flavors in RF cheeses (Olson, 1990). The proteolytic adjunct culture added to the FC cheese did not have a positive effect on flavor or overall acceptance compared to the RF control cheese. The FC cheese was scored as bitter and high acid by trained panelists. The pH also continued to drop during aging. Low salt, low pH, and the mixed starter culture used in Viking cheese may have inhibited activity from this culture.

Decreasing the amount of starter and decreasing ripe time are two suggested methods to decrease or slow acid production (Olson, 1990). During the make-procedure for the DS and DR RF cheeses, these modifications slowed acid development in comparison to the RF control cheese. However, these individual modifications did not have an effect on pH development during aging. The DS RF cheese was not different from the RF control cheese according to the difference panel at 3 or 7 mo. The DR RF cheese received lower flavor scores than the RF control cheese, but was not different in overall acceptance scores from the control RF cheese at the 3 mo evaluation. After 7 mo, difference panelists could not detect differences between the DR RF cheese and the RF control cheese.

Evaluation of firmness and texture by the acceptance panel indicated differences for these attributes for the RF and FF control cheeses for both days at 3 mo but not at 7 mo. Firmness and textural differences, as well as defects, in RF and FF cheeses were pronounced by 7 mo. At 3 mo, most young cheeses exhibit a green and rubbery texture and untrained panelists may have experienced difficulty in differentiating between treatments or understanding the terms.

Acceptance panels showed good agreement with trained panels. Both FF and WC cheeses scored the highest flavor scores with dairy judges and the highest overall acceptance and flavor scores from the acceptance panels. Similarly, the H cheese received the lowest judging flavor scores and overall acceptance scores. Dairy judge flavor scores and acceptance panel flavor scores had a mean correlation of 0.46 and 0.89 at 3 and 7 mo, but dairy judge flavor scores and overall acceptance scores had a mean correlation of 0.92 and 0.97 at 3 and 7 mo.

CONCLUSIONS

MAKE-PROCEDURE MODIFICATIONS can result in RF cheeses that are different from RF cheeses made with a standard make-procedure. Washing the curd of RF cheeses with 35°C water for 5 min after dipping was an effective modification for maximum sensory quality of RF semi-hard cheeses. Homogenization of whole milk prior to mixing with skim milk for RF cheesemaking resulted in a cheese with consistently low sensory quality after 3 and 7 mo aging. Other modifications did not have a consistent and/or significant effect on sensory quality. Further research on additional modifications and their combinations may lead to additional methods to maximize sensory quality of RF cheeses.

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Viscoelastic Properties of Butter

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ABSTRACT

VISCOELASTIC PROPERTIES were studied on butters prepared from fractionated high-melting triglyceride (HMT) and anhydrous milk fat (AMF). The elastic moduli (G'), the viscous moduli (G") and the complex viscosities (η^*) were determined as functions of angular frequency (ω). With increasing frequency, η^* and G" decreased while G' increased. Higher G', G" and η^* values were obtained for HMT butter, indicative of its firmer characteristics. Shift factors, determined using method of reduced variables, followed the Arrhenius model, from which the energies of activation were calculated. Power law intercepts, determined by relating G* to ω, exhibited a semi-logarithmic inverse relationship with temperature. Slight increase in power law intercepts was observed on storage due to thixotropic rearrangements of fat crystals.

Key Words: butter, viscoelastic, flowability, elastic moduli, anhydrous milk fat

INTRODUCTION

STRUCTURALLY, BUTTER CONSISTS of a continuous liquid fat phase with fat globules, crystalline fat and aqueous phase dispersed in it (Juriaanse and Heertje, 1988). Under certain conditions, fat globules are capable of extreme elongations without rupture, indicating the elastic nature of the membrane that surrounds the globule and are, therefore, approximated by a Maxwell element. Liquid fat surrounding the globules acts as a viscous fluid and flows on application of stress (Diener and Heldman, 1968). Butter thus exhibits viscoelastic characteristics. The rheology of butter can not be understood adequately without determining its viscoelastic properties; yet efforts have been limited in this regard (Shama and Sherman, 1970; deMan and Gupta, 1985; Rohm and Weidinger, 1993).

Techniques used to study butter rheology have been mostly shear-based and insensitive to its crystalline fat network. High shear rates applied in such methods interrupt the structure being measured and introduce additional parameters of structure variations with time. Also, the viscoelastic parameters such as elastic and viscous moduli cannot be determined separately from such tests (Rohm and Weidinger, 1993). Dynamic mechanical analysis, a fundamental technique, provides the advantages of providing data at small shear rates, measurement of elastic as well as viscous components of viscosity, and small sample size requirement (Ferry, 1980). Such small deformation measurements of linear viscoelastic behavior are useful for inferring structural properties of "unperturbed" materials. The method, primarily developed for polymeric systems, has also been used in the food industry: Taneya et al. (1979) for natural and processed cheese; Wendy et al. (1985) for cake batters; Nolan et al. (1989) for natural and imitation mozzarella cheese; Tunick et al. (1990) for Cheddar and Cheshire cheeses. Rohm and Weidinger (1993) used this technique for testing butter, but the effects of storage and chemical composition were not studied. Shukla et al. (1994) studied the effect of chemical composition on viscoelastic properties of butter samples. Our objective was to investigate dynamic properties of butter by studying the effects of time and temperature of storage on the complex mod-

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ulus and determining activation energies by fitting Arrhenius models to shift factors.

MATERIALS & METHODS

Materials

Fresh high-melting triglyceride (HMT) and anhydrous milk fat (AMF) butter samples were made using the recombination method. Composition was in compliance with the definition of butter (80.5% fat, 16.0% water, salt 1.5% and skimmed milk powder 2.0%). Manufacturing procedures and physicochemical properties of butters have been described (Shukla et al., 1994). Samples were immediately stored at different temperatures (17, 22 and 27°C) and analyzed periodically for rheological changes.

Dynamic mechanical analysis

Small amplitude oscillatory measurements were performed on a Bohlin VOR rheometer (Bohlin Instruments Inc., Cranbury, NJ) using a 90 g-cm torsion bar and parallel plate geometry (15 mm plate diameter and 4 mm plate gap). Cork borers of 15 mm internal radii were used to produce cylindrical butter samples. Appropriate height was cut from these cylinders and placed carefully on the lower plate. The sample was then allowed to rest to decay the compressive force and to attain temperature equilibrium. A corrugated top plate was used to avoid slippage of samples. Four test temperatures were used in the range 12–32°C. Strain-sweep tests were conducted at frequency of 1 Hz to determine the linear viscoelastic range. The complex viscosities (η^*), the storage modulus (G'), the loss modulus (G"), and the complex modulus (G*) were determined between frequencies 0.628–62.8 rad/sec at low strains (<0.1%) in the linear range. Results were reported as averages of three replicates. Power law indices were determined for complex modulus (G*) using the following relationship (Nolan et al., 1989):

$$G^* = G_0 {}^* \omega^{n^*} \tag{1}$$

RESULTS & DISCUSSION

STRAIN SWEEP PROFILES were first obtained to determine the linear range where viscoelastic properties were independent of strain rates (Fig. 1). A very small linear range (< 0.1%) was
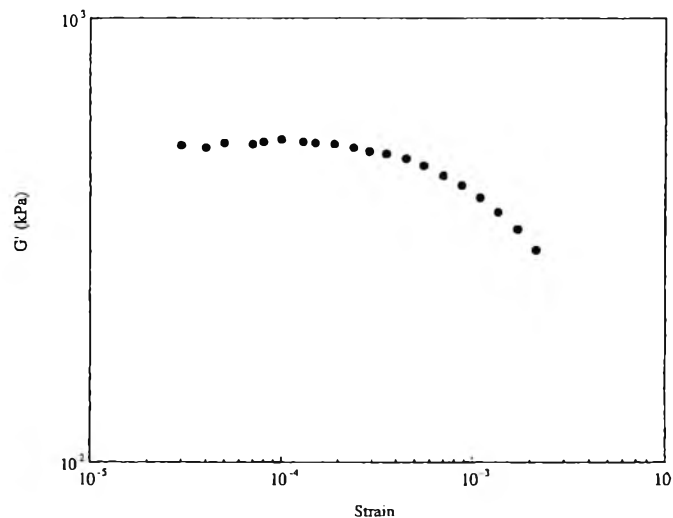
Fig. 1—Strain sweep profile of a representative butter sample at a frequency of 1 Hz.

observed for both butter samples at all temperatures. At higher strain levels, the moduli decreased. These small critical strain levels were observed probably due to destruction of weak van der waals forces between fat crystals at higher strains. Similar results have been reported by Rohm and Weidinger (1993).

Frequency sweep profiles were determined in this linear range for later studies (Fig. 2) of HMT butter sample at 32°C and AMF butter at 22°C. These temperatures were selected since viscoelastic functions of samples were similar at these temperatures. For both the samples, η^* and G" decreased while G' increased with increasing frequency. Similar spectra for both samples revealed existence of similar 3-dimensional networks in both samples. G' and G" showed little frequency dependence, indicative of the presence of a network with low possibility of rupture of junction zones within the low-frequency range used. Similar observations were made by Doublier and Choplin (1989). The G' increased whereas the G" decreased with increasing frequency, showing a change from relatively more viscous-like to more elastic behavior. Rohm and Weidinger (1993) also observed similar behavior for butter. The values of G' were higher than G" at any given frequency due to greater contribution of the elastic component (G'), to the dynamic viscosity, than of the viscous component (G"). Since G' represents solid behavior of a material, higher G' values show that butter is a viscoelastic solid. Similar patterns were reported for other foods: Mozzarella cheese (Glymph, 1994); gels (Doublier and Choplin, 1989); and Cheddar cheese (Tunick et al., 1990).

In order to study the effects of temperature, frequency sweep profiles were determined over a range of temperatures commonly employed in the use of butter. The values of G' and G* obtained at different temperatures were plotted against angular frequency on logarithmic scale (Fig. 3 and 4). In earlier studies for butter, such spectra have not been reported for G* (Shukla et al., 1994; Rohm and Weidinger, 1993; Borwankar et al., 1992). With increasing temperature, these viscoelastic parameters decreased over the frequency range used due to melting of crystalline fat. Similar temperature-effect was observed for complex viscosities and flow curves had similar slopes but different intercepts (Shukla et al., 1994). The change in temperature seemed to shift the curves sideways. The degree of shift was determined as 'shift factor', a_T, and was calculated for complex viscosities using the time-temperature superposition principle (Shukla et al., 1994).

For each sample, a plot of ln a_T vs reciprocal of absolute temperature (1/T) produced a straight line which followed the Arrhenius equation:

$$a_T = A \exp(-E/RT) \qquad (2)$$

where A is the pre-exponential factor, E is the activation energy, and R is the gas constant (1.987 cal/°C mol). Activation energies were determined as slopes multiplied by R. The Arrhenius equations and activation energies of both samples were compared (Table 1). Higher activation energies and slopes depict greater shift in η^* with temperature for HMT butter. Although Arrhenius fit for shift factors has been used, activation energies were not calculated (Whorlow, 1980). Tunick et al. (1990) used the Arrhenius model for complex viscosities of Cheddar cheese and reported activation energy as 32.8 kcal/mol.

In order to describe the effects of temperature, complex modulus (G_0^*) was selected as the rheological parameter as it is indicative of degree of firmness of samples (Borwankar et al., 1992). Power law model was fitted to relate G* to ω and, slope and intercept values were obtained from the relationship, $G^* = G_0^* \omega^{n*}$. Regression coefficients, r^2, were > 0.98 for both butter samples, indicating goodness of fit. The power law intercepts, G_0^*, represent the values of G* at an angular frequency of 1 rad/sec. Temperature correlated negatively with G_0^* (Table 2). Semi-logarithmic plots of G_0^* vs temperature were drawn and an exponential relationship was observed (Fig. 5). The resulting regression equation coefficients derived from such linear trans-
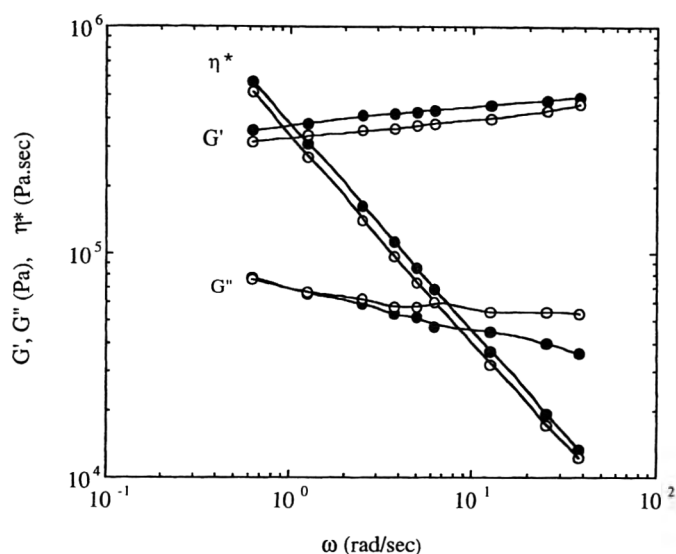
Fig. 2—Dynamic mechanical analysis frequency sweep profiles for representative butter samples (● = HMT butter at 32°C, ○ = AMF butter at 22°C).
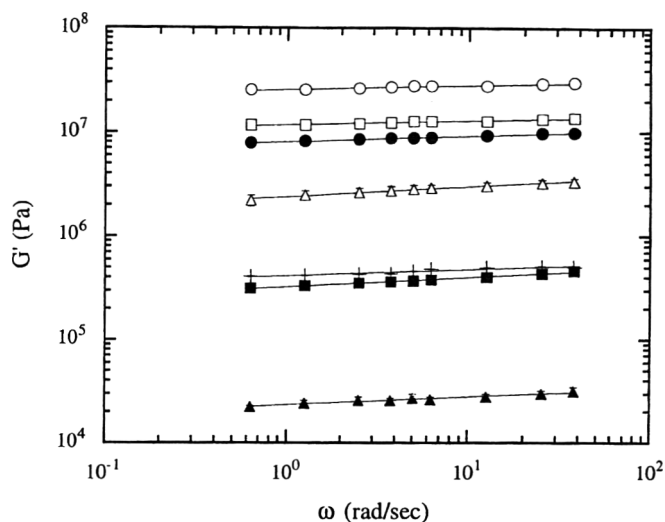
Fig. 3—Storage modulus vs frequency of butter samples at different temperatures (● = AMF butter at 17°C, ■ = AMF butter at 22°C, ▲ = AMF butter at 27°C, ○ = HMT butter at 17°C, □ = HMT butter at 22°C, △ = HMT butter at 27°C, + = HMT butter at 32°C).

formation were compared (Table 3). The higher slope value for AMF butter indicates its enhanced temperature dependency in comparison with HMT butter.

Temperature effect could be explained on the basis of solid fat contents of samples. In plastic products like butter, rheological properties are determined mainly by the degree of crystallinity of the milk fat they contain (Rohm and Weidinger, 1993). Milk fat, the major ingredient in butter, is a mixture of various triglycerides with melting points varying from −40 to +40°C. At a specific temperature, part of it exists in the crystalline state and imparts solid-like elastic characteristics to the product (Bailey, 1950). As the temperature is increased, melting of crystalline fat takes place resulting in more liquid-like behavior of samples as we observed. The effects of temperature on G_0^* were found to be significant (p < 0.01). When compared with AMF butter, HMT butter had higher values of G_0^* at similar temperatures (Table 2). Differences in rheological behavior of butter samples could be attributed to their distinct chemical composition. HMT butter being richer in high-melting triglycerides
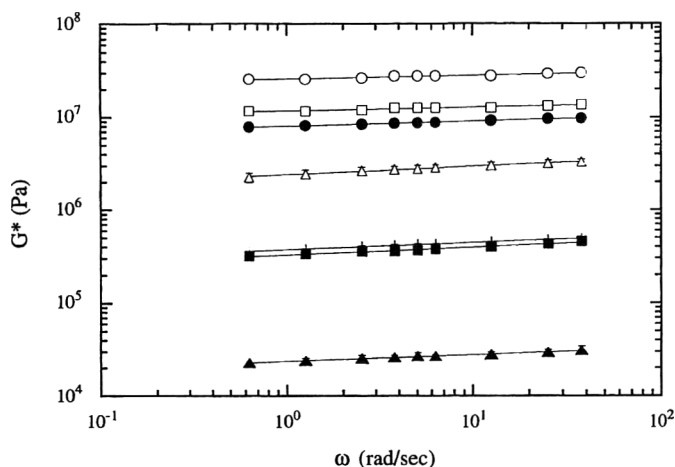
Fig. 4—Complex modulus vs frequency of butter samples at different temperatures (● = AMF butter at 17°C, ■ = AMF butter at 22°C, ▲ = AMF butter at 27°C, ○ = HMT butter at 17°C, □ = HMT butter at 22°C, △ = HMT butter at 27°C, + = HMT butter at 32°C).

Fig. 5—Power law intercept for G* (Go*) as a function of temperature for butter samples. ● = HMT butter, ▲ = AMF butter.

Table 1—Arrhenius equation and activation energies for shift factors (a_T) of the high-melting triglyceride (HMT) and anhydrous milk fat (AMF) butter samples

Sample	Arrhenius equation (ln a_T =)	Activation energy (kcal/mol)
AMF butter	57609/T − 192.27	114.47
HMT butter	26513/T − 88.02	52.68

Table 2—Power law parameters for G* of high-melting triglyceride (HMT) and anhydrous milk fat (AMF) butter samples at different temperatures

		Power law parameters	
Sample	Temperature (°C)	Slope (n^*)	Intercept Go* (MPa.s^{n*})
AMF butter	17	0.06	8.02
	22	0.09	0.32
	27	0.08	0.02
HMT butter	17	0.04	25.76
	22	0.04	11.73
	27	0.09	2.38
	32	0.06	0.41

Table 3—Regression equation coefficients from the relationship Go* = a b^T for high-melting triglyceride (HMT) and anhydrous milk fat (AMF) butter samples

		Coefficients	
Sample	a × 10^{-9}	b	r^2
AMF butter	4.43	0.75	0.97
HMT butter	147.5	0.56	0.99

Fig. 6—Relationship between complex modulus (G*) and liquid fat content for high-melting triglyceride (HMT) and anhydrous milk fat (AMF) butter samples. ● = HMT butter, ▲ = AMF butter.

had greater amounts of crystalline fat (Shukla et al., 1994) which resulted in its more solid-like behavior. The difference in the two samples was significant (p < 0.001).

Since the liquid fat content affected the flow behavior of butter, dynamic mechanical results were related to the amount of liquid fat and to temperature. Complex modulus values obtained at a frequency of 1 rad/sec at different temperatures were plotted vs liquid fat contents at those temperatures (Fig. 6). Values of liquid fat contents were determined using differential scanning calorimetry and were reported previously (Shukla et al., 1994). Complex modulus values decreased exponentially with increases in liquid fat content for both samples indicating loss of firmness, as expected. However, slopes of curves for the 2 samples were different. Higher slope for AMF butter indicated greater sensitivity of G* values to changes in liquid fat content. A possible reason for such behavior may be the different chemical composition of the liquid fat fraction of the 2 samples. It has been

reported that the viscosity of fats was affected by the degree of unsaturation and fatty acid chain length (Swern, 1964). HMT butter had higher concentrations of long-chain unsaturated fats and lower contents of short-chain saturated fatty acids (Shukla et al., 1994). Liquid fats in the 2 samples at any temperature were thus enriched with different types of fatty acids which might have caused different G* values for the same liquid fat content.

The effects of storage on viscoelastic properties of the 2 samples was studied. Power law intercepts for complex modulus (G₀*) showed an increasing trend on storage for both the samples, indicating a higher degree of firmness of samples due to thixotropic rearrangement of the crystals into a 3-dimensional scaffolding network on storage (Table 4). In fresh butter, fat exists as loose crystal chains which slowly form ramifications due to weak van der waals attraction forces on setting. This crystal network is later reinforced by strong primary bonds slowly formed between the larger crystals resulting in harder samples (Haighton, 1965). Storage effect was insignificant (p > 0.05).

CONCLUSION

VISCOELASTIC PROPERTIES of butter were determined using dynamic mechanical analysis, which were dependent upon strain

Temp (°C)	HMT butter				AMF butter			
	0	10	20	28	0	7	14	21
17	25.76	26.56	26.78	27.67	8.02	11.78	12.17	13.34
22	11.73	12.02	12.22	12.51	0.32	0.32	0.32	0.35
27	2.38	2.52	2.53	2.55	0.02	0.02	0.02	0.03

level, frequency, temperature, storage time and chemical composition of samples. Dynamic mechanical analysis was successfully employed to compare viscoelastic characteristics of HMT and AMF butter samples. HMT butter indicated more solid-like behavior in comparison with AMF butter at similar temperatures as shown by its higher power law intercepts. At 32°C, it had similar viscoelastic parameters as AMF butter at 22°C. Temperature had a negative correlation with rheological parameters. On storage, increased firmness of samples was observed probably due to crystalline network rearrangements.

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Heat Denaturation and Emulsifying Properties of Egg Yolk Phosvitin

SIEW LIAN CHUNG and LES K. FERRIER

ABSTRACT

Phosvitin in water at pH 7 had a denaturation temperature (T_d) of 79.7 ± 1.4°C when heated at 10°C/min. When dissolved in 0.1M and 1.0M NaCl, the T_d decreased to 77.7 ± 1.2°C and 77.2 ± 1.3°C, respectively, and in 10 and 20% sucrose there was no change in T_d. Heat treatment of phosvitin solutions at ≥65°C led to decreased emulsifying activity (EA). The emulsion stability (ES) decreased when phosvitin solutions were heated at 70, 80 or 96°C for up to 60 min. The ES was not affected ($p < 0.05$) for phosvitin solutions after heating at ≤67.5°C for up to 60 min.

Key Words: egg yolk, phosvitin, heat denaturation, emulsification

INTRODUCTION

HEAT TREATMENTS such as pasteurization and spray-drying of egg products are widely used to ensure safety and extend shelf-life. However, heat treatment of foods normally results in protein denaturation and degrades functionalities such as emulsifying properties. Secondary, tertiary or quaternary egg protein structure may be disrupted depending on the temperature and time of heating. Most food proteins display functional properties only within a narrow temperature range (Cheftel et al., 1985). Chang et al. (1970a), using disc gel electrophoresis, reported that some egg yolk proteins, e.g., α- and β-livetin, were sensitive to pasteurization at 61.7°C for 3 min. Denaturation of proteins changes physical properties such as viscosity and solubility, as well as emulsifying properties. The viscosities of unheated native egg yolk and yolk heated at 65.6°C for 3 min were 23 and 200P at a shear rate of 1.9 sec^{-1}, respectively (Chang et al., 1970b) due mainly to the formation of lipoprotein complexes.

Phosvitin, the major phosphoprotein in egg yolk, has been separated into two components designated α- and β-phosvitin by gel filtration (Abe et al., 1982). Heat-resistant phosvitin was first reported by Mecham and Olcott (1949). Phosvitin solutions at "pH from 4 to 8 when heated for several hours at 100°C did not precipitate nor show any other evidence of change" (Mecham and Olcott, 1949). Itoh et al. (1983) reported no change in the electropherogram of an α- and β-phosvitin solution heated below 100°C for 10 min, but the phosvitin bands were completely diffused after heating at 140°C. However, Dixon and Cotterill (1981) reported that the phosvitin bands in polyacrylamide gels disappeared when egg yolk was heated above 72°C. No report is available on the heat denaturation temperature (T_d) of phosvitin as determined by differential scanning calorimetry (DSC). We determined the T_d from the endotherm of the DSC curves during unfolding of protein molecules. The effects of heat on the functional properties, such as emulsification, of phosvitin have not been reported. Our objectives were to study the heat stability and the emulsifying properties of phosvitin after heat treatment of phosvitin solutions.

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MATERIALS & METHODS

Materials

Phosvitin was purchased from Sigma Chemical Co. (St. Louis, MO). 1-Menthol and α-naphthol were obtained from Fisher Scientific Co. (Fair Lawn, NJ). Indium was provided by DuPont Instruments (Wilmington, DE).

Differential scanning calorimetry (DSC)

DSC thermograms were recorded on a DuPont DSC 2910 instrument (Wilmington, DE). Indium was used to calibrate the cell constant. Temperature calibrations were performed using 1-menthol, α-naphthol and indium. Phosvitin was dissolved in distilled water at a concentration of 15% and adjusted to pH 7 with 1.0 M HCl. Phosvitin solutions (25 μL) were hermetically sealed in aluminum pans and heated at rates of 5, 10, 20 and 30°C/min. Distilled water was used in the reference pan. The phosvitin solutions were heated from 25 to 140°C with continuous scanning. Heat denaturation temperatures (T_d) were determined from the peak of the endotherm estimated using DuPont program General 4.1. The effects of concentrations of 0.05, 0.1 and 1.0 M NaCl, and 10 and 20% sucrose on T_d were studied (five replicates).

Heat treatment of phosvitin solutions

Samples (11 mL) of 0.5% phosvitin solutions at pH 7 were placed in 2.5-cm-diam screw-cap vials and heated to 40, 60, 65, 67.5, 70, 80, and 96°C in a water bath. The temperature fluctuation was ± 0.5°C. Vials were removed from the water bath 10, 20, 40 and 60 min after reaching the desired temperature and immediately immersed in ice water.

Emulsifying activity (EA) and emulsion stability (ES)

Emulsions were prepared, using the heat-treated phosvitin solutions, as described by Chung and Ferrier (1991a). EA was assayed with the turbidimetric method of Pearce and Kinsella (1978) as modified by Chung and Ferrier (1991a).

Emulsions were held at 23°C for 24 hr and column heights (representing volume) of the lower aqueous layer, middle (emulsion) layer and the upper oil layer were measured to the nearest 0.5 mm. Emulsion instabilities were expressed as percentage (v:v) of drainage (lower aqueous layer) and coalescence (upper oil layer) relative to total volume. The ES was calculated as the percentage (v:v) of emulsion layer left after standing for 24 hr (Chung and Ferrier, 1992). Triplicate analyses were done.

Kinetic parameters from DSC

The statistical analysis was based on that of Ozawa (1970) who developed the relationship between T_d and DSC heating rate, B:

$$\ln(B/T_d^2) = -E_a/RT_d + \ln(AR/E_a) \qquad (1)$$

where R is the ideal gas constant, E_a is the activation energy of protein denaturation and A is the pre-exponential factor. Data were examined by means of the least squares regression of $\ln(B/T_d^2)$ vs $1/T_d$, using the SYSTAT program (Wilkinson, 1986). E_a was obtained from the slope of the regression straight line. The values of E_a and A were determined by the least squares method which satisfied the assumption of homogeneity of variance in the residuals across the values of the independent variable, $1/T_d$.

Analysis of loss of EA with heating temperature and time

The rate of decrease in EA followed a first-order kinetic equation:

$$\ln EA = \ln EA_o - k\,t \qquad (2)$$

where EA_o is the initial EA, k the rate constant and t the heating time.
The Arrhenius equation (Eq. 3) described the influence of heating temperature, T, on k:

$$k = A\,\exp(-E_a/RT) \qquad (3)$$

The rate constant, k, was obtained from regression of ln EA vs t from Eq. (2). The value of E_a was obtained from subsequent regression of ln k vs 1/T from Eq. (3).

Analysis of variance (ANOVA) was performed using SYSTAT at the 5% significance level (Wilkinson, 1986).

RESULTS & DISCUSSION

IN A CHARACTERISTIC THERMOGRAM of phosvitin in water and NaCl solutions at pH 7 (Fig. 1) the baselines of the endotherm were not apparent. The endothermic change (heat uptake) of phosvitin was gradual with temperature and a broad peak was produced, indicating that a change in phosvitin structure began from the start of heating. The T_d was 79.7 ± 1.4°C for phosvitin in water at a heating rate of 10°C/min. The T_d for phosvitin increased linearly with heating rate (Fig. 2). Increasing the heating rate from 5 to 30°C/min raised the apparent denaturation temperature by 11°C. Protein denaturation is described by first-order kinetics (Cheftel et al., 1985). Phosvitin was not stable in this experiment at 100°C, contrary to reports by Mecham and Olcott (1949). Dixon and Cotterill (1981) observed that the electrophoretic bands for phosvitin in egg yolk disappeared in the range 69 to 72°C, which is closer to the T_d values studied under different heating rates we used. The activation energy for heat denaturation of phosvitin was 164 kJ/mol, whereas larger activation energies were reported for ovalbumin (552 kJ/mol) and peroxidase (773 kJ/mol) (Cheftel et al., 1985). The smaller activation energy indicates that phosvitin denaturation is less sensitive to temperature change than ovalbumin or peroxidase.

The T_d (at 10°C/min) of phosvitin decreased to 77.7 ± 1.2°C and 77.2 ± 1.3°C (Fig. 1) when phosvitin was dissolved in 0.1 and 1.0M NaCl, respectively, but the T_d did not change in 0.05M NaCl as compared to that of water. The T_d between phosvitin in 0.1 and 1.0M NaCl were not different.

The T_d for phosvitin did not change in 10 or 20% sucrose solutions, as compared to dissolution in water. Dixon and Cotterill (1981) reported that neither NaCl nor sucrose protected phosvitin from heat denaturation. Nevertheless, glucose, sucrose, lactose, and NaCl slowed the unfolding of egg white, milk and whey proteins and, therefore, shifted the T_d to a higher temperature (Donovan et al., 1975; Itoh et al., 1976; de Wit, 1981).

Emulsifying activity and emulsion stability

The pH of heat-treated phosvitin solutions remained unchanged at pH 7 after heating at 40–96°C. Heating phosvitin solutions at 40 and 60°C for up to 60 min did not decrease the EA compared to the control which was not heat-treated and maintained at 23°C (Fig. 3). However, the EA decreased after the phosvitin solutions were heated at ≥65°C. This indicated that α- and β-phosvitin were slowly denatured above 60°C. Statistical analysis indicated that the interaction effects between temperature and time were significant for phosvitin solutions heated at 65–96°C. Kinetic analysis indicated that the rate of loss in EA was first-order and increased with heating temperature. Raising the temperature from 65 to 70°C increased the rate constant 3.3 times (Fig. 4). However, raising the temperature from 70 to 96°C increased the rate constant only 1.3 times (Fig. 4). The results indicated a large decrease in EA over the range 65–70°C. There was a change of slope of the Arrhenius plot at 70°C (Fig. 4). From 65 to 70°C, the activation energy, E_a, was 225 ± 26
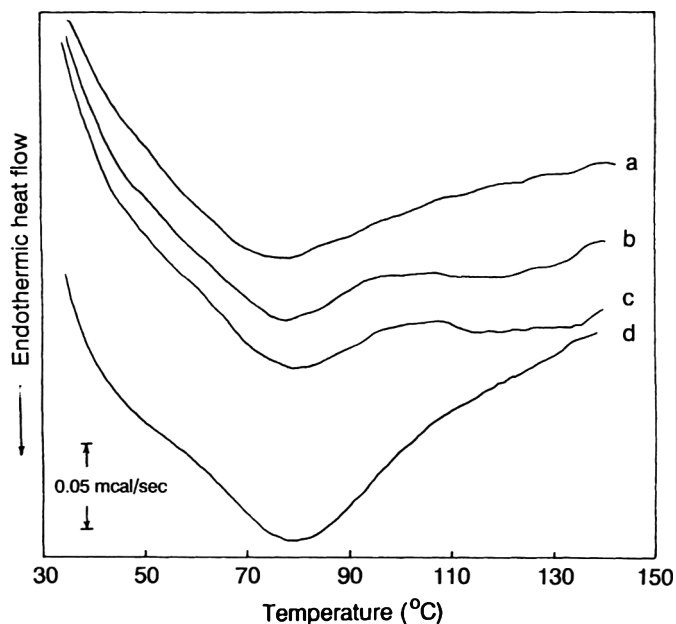
Fig. 1—DSC thermograms for phosvitin in (a) 1.0M NaCl, (b) 0.1M NaCl, (c) 0.05M NaCl, and (d) distilled water. 15% phosvitin solutions at pH 7 heated at 10°C/min.
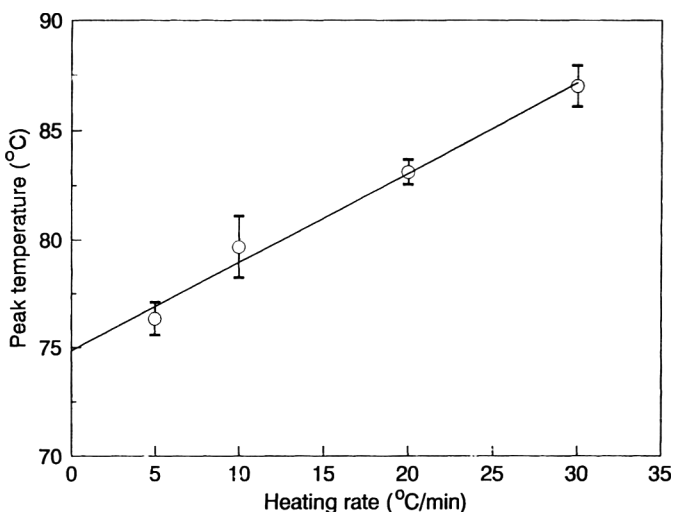
Fig. 2—Heating rate and denaturation temperature of 15% phosvitin solutions in water at pH 7. $T_d = 74.85 + 0.41B; r^2 = 0.941$. Vertical bars indicate standard deviations (B = DSC heating rate).

kJ/mol, and from 70 to 96°C, E_a was 12 ± 1 kJ/mol. Thus, the rate constants for 65–70°C were more dependent on temperature than those for 70–96°C. The change of E_a from the temperature range 65–96°C could be due to a two-stage denaturation mechanism since protein denaturation may follow several different reaction paths (Joly, 1965). For example, denaturation of lysozyme was described by a two-state mechanism, where native protein was first changed to an intermediate state with different partially denatured conformations before a denatured state (Ahmad et al., 1983).

Heating at 70, 80 or 96°C for 60 min decreased the ES from 69 to 56, 47 and 42%, respectively (Fig. 5). Heating phosvitin solutions at ≤67.5°C for up to 60 min did not decrease the ES. Extended heating at ≥70°C may aggregate the unfolded protein molecules and decrease solubility. Soluble egg yolk protein is a major contributor to emulsification (Chung and Ferrier, 1991b). Aqueous layer separation occurred in all the phosvitin emulsions examined (Table 1). For phosvitin solutions heated at 70°C for 40 and 60 min, and all those heated at 80 and 96°C, coalescence
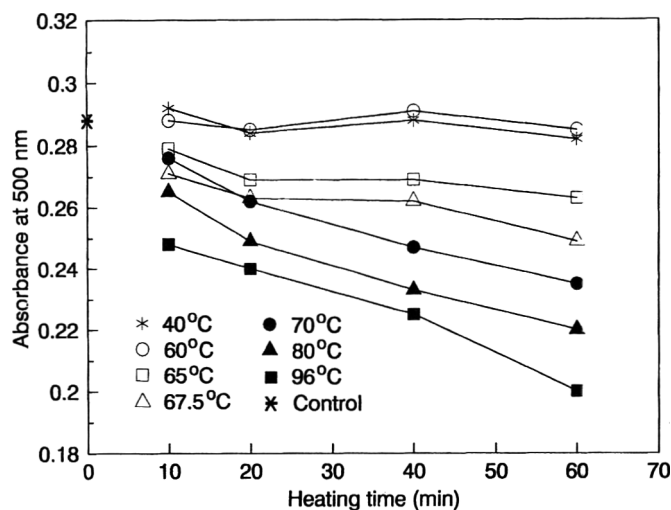
Fig. 3—Heating time and emulsifying activity of phosvitin at different heating temperatures.
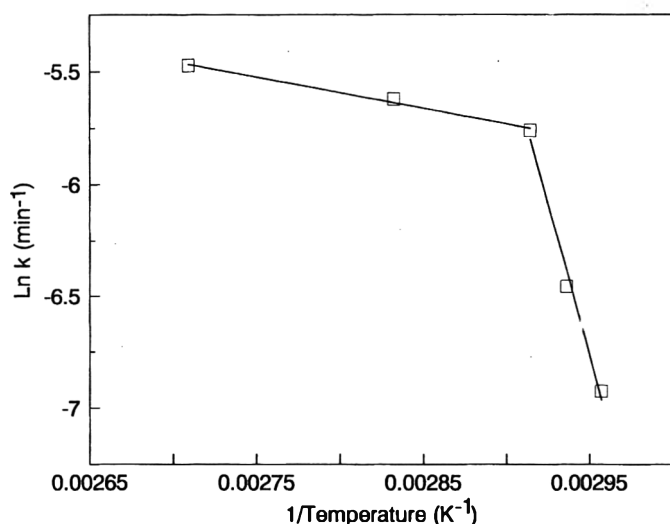
Fig. 4—Relationship of rate constant and heating temperature for emulsifying activity of phosvitin between 65 and 96°C.
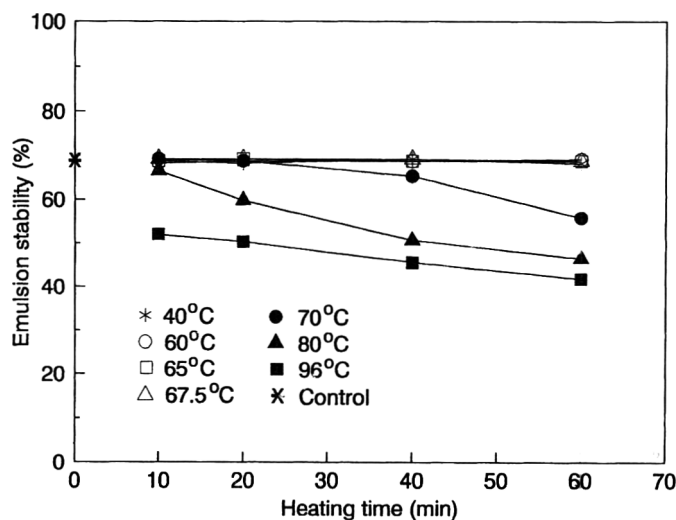
Fig. 5—Heating time and emulsion stability of phosvitin at different heating temperatures.

Table 1—Aqueous and oil layer separations for phosvitin emulsions

Temp (°C)	Time (min)	Aqueous layer[a] % (v/v)	Oil layer[a] % (v/v)
Control		31.2 ± 0.8	0
40	10	31.2 ± 0.8	0
	20	31.7 ± 1.5	0
	40	30.8 ± 1.3	0
	60	31.7 ± 0.8	0
60	10	31.7 ± 1.5	0
	20	31.2 ± 0.8	0
	40	31.2 ± 0.8	0
	60	30.8 ± 0.0	0
65	10	31.2 ± 0.8	0
	20	30.8 ± 1.3	0
	40	31.2 ± 0.8	0
	60	31.2 ± 0.8	0
67.5	10	30.8 ± 0.0	0
	20	30.8 ± 1.3	0
	40	30.8 ± 0.0	0
	60	31.2 ± 1.5	0
70	10	30.8 ± 0.0	0
	20	31.2 ± 0.8	0
	40	31.7 ± 0.8	3.0 ± 0.8
	60	37.2 ± 1.3	6.9 ± 1.4
80	10	32.1 ± 1.3	1.3 ± 0.0
	20	36.3 ± 0.8	3.9 ± 1.3
	40	44.1 ± 0.8	5.2 ± 1.3
	60	45.4 ± 0.7	8.1 ± 0.8
96	10	42.8 ± 4.1	5.2 ± 1.3
	20	42.8 ± 0.8	6.8 ± 0.8
	40	47.1 ± 0.8	7.3 ± 0.8
	60	47.9 ± 0.8	10.3 ± 1.3

[a] Number following the ± sign is the standard deviation.

of oil droplets occurred in the emulsions; coalescence increased with heating time (Table 1).

CONCLUSIONS

SODIUM CHLORIDE but not sucrose, decreased the T_d of phosvitin solutions at pH 7. The apparent T_d depended on heating rate of DSC. Emulsions prepared from heat-treated phosvitin solutions were stable but lost stability after phosvitin solutions were heated at ≥70°C. The loss in emulsifying property depended on the heating temperature, time and interaction effects of temperature and time. Phosvitin remains stable during normal pasteurization temperature, but would denature at typical cooking or sterilization temperatures.

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Antioxidant Inhibition of Cholesterol Oxidation in a Spray-Dried Food System during Accelerated Storage

K.C. HUBER, O.A. PIKE, and C.S. HUBER

─────────── ABSTRACT ───────────

Spray-dried egg yolk was used to evaluate antioxidant inhibition of cho-
lesterol oxidation during accelerated (Cu^{+2} and heat) storage. Liquid yolk
was treated with equimolar amounts of BHA (0.01%, w/w of lipid),
ascorbyl palmitate (0.023%), or a tocopherol blend (0.023%). Yolk
batches were spray-dried, stored at 60 ± 2°C for up to 28 days, and
analyzed for cholesterol oxidation products (COPs) using gas chroma-
tography. COP levels generally increased during storage with 7-keto-
cholesterol predominant. Significant antioxidant effects were manifest in
decreased levels of 7-ketocholesterol, 7α- and 7β-hydroxycholesterol;
while cholestane-triol and cholesterol-5,6-epoxide levels were not af-
fected. All antioxidants showed significant inhibitive effects relative to
the control, and tocopherols were more effective than ascorbyl palmitate.

Key Words: egg yolk, cholesterol oxides, antioxidants, ascorbyl palmi-
tate, tocopherols

INTRODUCTION

CHOLESTEROL OXIDATION PRODUCTS (COPs) have received con-
siderable attention due to their biological activities associated
with human disease. Research provides evidence some choles-
terol oxides are toxic and may facilitate development of coro-
nary artery disease and certain cancers (Peng et al., 1992a, b;
Morin and Peng, 1992; Morin et al., 1992).

Because of such health concerns, the presence of cholesterol
oxides in food products has been extensively investigated. COPs
have been detected in commodities including dairy, egg, meat,
and other animal foodstuffs which are commonly subjected to
elevated processing temperatures, pro-oxidative conditions or
storage (Tsai and Hudson, 1984, 1985; Lee et al., 1985; Missler
et al., 1985; Park and Addis, 1985a; Nourooz-Zadeh and Ap-
pelqvist, 1987, 1988; van de Bovenkamp et al., 1988; Sander et
al., 1989; Zhang et al., 1991). Research has confirmed the ten-
dency of COPs to increase during storage (Missler et al., 1985;
Tsai and Hudson, 1985; Morgan and Armstrong, 1987).

Since cholesterol oxidation proceeds via a free radical mech-
anism, antioxidants used to inhibit general lipid oxidation may
also retard cholesterol oxidation (Smith, 1980). Concern regard-
ing the safety of synthetic antioxidants has prompted investi-
gations of the effectiveness of naturally occurring compounds
(Branen, 1975; Anonymous, 1986; Addis and Park, 1992).

Studies involving application of antioxidants to inhibit cho-
lesterol oxidation have been limited. BHA, BHT and propyl
gallate, common synthetic antioxidants, were slightly effective
in slowing hydrogen peroxide-induced cholesterol oxidation in
egg yolk during spray-drying (Morgan and Armstrong, 1987).
Using an aqueous model system, Rankin and Pike (1993) eval-
uated several novel antioxidants for preventing cholesterol oxi-
dation. Only tocopherol isomers proved effective in delaying
cholesterol autoxidation. However, whether the results using this
model system have widespread application to food systems is
unknown. In addition, Wahle et al. (1993) incorporated α-to-
copherol at various concentrations into the feed of hens, pro-
ducing several α-tocopherol levels in eggs. The whole egg was
spray-dried and stored at ambient temperature exposed to air

and light. During storage, an inverse relationship between COP
formation and α-tocopherol content was observed.

Spray-dried, cholesterol-containing foods, subjected to air and
heat during processing, are susceptible to sterol oxidation during
preparation and storage, and therefore might benefit from anti-
oxidant addition. Spray-dried egg products have been utilized in
cholesterol oxidation research because of their high cholesterol
content and pro-oxidative processing conditions. (Morgan and
Armstrong, 1987; Sander et al., 1989; Morgan and Armstrong,
1992). Our objective was to evaluate the effectiveness of several
antioxidants in inhibiting cholesterol oxidation during acceler-
ated (Cu^{+2}, heat) storage of spray-dried egg yolk.

MATERIALS & METHODS

Antioxidant preparation

Stock solutions (0.135 M) of ascorbyl palmitate (Hoffman La Roche,
Inc., Nutley, NJ), BHA (Sigma Chemical Co., St. Louis, MO), and
Tenox GT-2 (Eastman Chemical Co., Kingsport, TN) were prepared in
ethanol. Tenox GT-2 is a commercially available tocopherol blend com-
prised of α- (15-20%), γ- (15-20%), and δ- (60-65%) isomers. Prepa-
ration of Tenox GT-2 molar stock solution was based on its 70%
tocopherol content and assumed an average tocopherol molecular weight
of 416.67 g/mole.

Liquid egg yolk preparation

Fresh, large grade A eggs were obtained from the Brigham Young
University Poultry Facility (Provo, UT). Egg yolks were separated from
albumen, pooled, blended 5 min at speed setting 1 with a Hobart A-
120T mixer (Hobart. Troy, OH), and stored at 4°C.

Blended yolk was divided into 500g batches. Treatment yolk batches
received equimolar amounts of BHA, ascorbyl palmitate, or the tocoph-
erol blend which resulted in addition levels of 0.01, 0.023, and 0.023%
(w/w), respectively, based on reported values for total yolk lipid (Cook
and Briggs, 1986). Control batches received no antioxidant. Equimolar
quantities of cupric sulfate were also incorporated into all liquid yolk
batches providing a final added concentration of ≈ 1.5 mg Cu^{+2}/g spray-
dried yolk.

Egg yolk batches were prepared by blending 0.3990 g anhydrous cu-
pric sulfate and 700 μL of antioxidant stock solution (pure ethanol for
control batches) with 158 mL of distilled water in a stainless steel mixing
bowl. Liquid yolk (500 g) was added and the mixture blended 15 min
at speed setting 1 with a Hobart N50 mixer (Troy, OH). Egg yolk di-
lution with distilled water served both to optimize the spray-drying pro-
cess as well as minimize contact between yolk protein and ethanol
(Morgan and Armstrong, 1992). After blending, treatment and control
batches were stored at 4°C until spray-dried.

Spray-dryer Operation

Treatment and control batches were dried using a Niro Mobile Minor
laboratory spray-dryer (Niro, Inc., Columbia, MD) equipped with an
electric air heater, a 24-vaned centrifugal atomizer, and a peristaltic
pump for delivering liquid yolk to the atomizer. Inlet air temperature
was set at 225°C while exhaust temperature was maintained at 100°C by
adjusting the flow rate of liquid yolk to the atomizer.

Accelerated storage

Experimental and control dried yolk batches were each divided into
1g samples. They were placed in 20 dram vials secured with Teflon-
lined screw caps and stored in a Lab-Line Imperial II radiant heat drying

oven (Lab-Line. Melrose Park, IL) at 60 ($\pm$ 2) °C for 0, 6, 14, 21, or 28 days. Following elevated temperature storage, samples were held at −20°C until analyzed.

Cholesterol oxide extraction and isolation

Lipid was extracted from dried yolk according to the procedure of Morgan and Armstrong (1989). Dried yolk (1g) was transferred from a 20 dram storage vial to a 250 mL round bottom flask, followed by addition of 100 mL reagent grade chloroform/methanol (2:1, v/v) for lipid extraction. The 20 dram vial was rinsed with 3 successive 3 mL aliquots of chloroform/methanol which were also added to the 250 mL flask. Twenty-five µg of 5-pregnen-3β-ol-7,20-dione (7-ketopregnenolone, Steraloids, Inc., Wilton, NH) in chloroform/methanol was added as an internal standard. The mixture was filtered through a 0.2 µm nylon membrane to retain yolk protein and the filtrate containing the lipid fraction was collected in an evaporation flask. An additional 25 mL of chloroform/methanol was used to rinse the extraction flask and wash the filter residue. Filtrate solvent was removed under vacuum at 40°C using a Büchi 001 Rotavapor (Büchi, Flawil, Switzerland).

The lipid extract was redissolved in 5 mL of hexane and applied to a Sep-Pak[*] silica cartridge (Waters Chromatography Division, Milford, MA) which had been wetted with 10 mL of hexane. The evaporation flask was rinsed with 3 mL and then with 2 mL of hexane and rinsings were also loaded onto the Sep-Pak. Elution of COPs was subsequently performed as outlined by Morgan and Armstrong (1989). COPs were eluted into a test tube with the acetone fraction, which was evaporated to dryness under nitrogen using a Meyer N-EVAP, model 111, analytical evaporator (Organomation Associates, Inc., Berlin, MA). Water bath temperature was maintained at 40°C. The dried extract was redissolved in ethyl acetate (850 µL) and transferred to a tapered 1.1 mL microvial for evaporation under nitrogen. This transfer/evaporation process was repeated two more times to ensure transfer of COPs from the test tube to the microvial.

The dried extract was redissolved in pyridine (50 µL) and BTZ (50 µL) (Supelco, Inc., Bellefonte, PA) and capped with a teflon-lined crimp cap. After addition of BTZ, samples were allowed to stand 15 min before GC injection (Park and Addis, 1985b).

Cholesterol oxide determination by gas chromatography

Gas chromatography was performed similar to the method of Park and Addis (1985b) using a Hewlett Packard (HP) 5890 gas chromatograph equipped with a HP 7673 autosampler and flame ionization detector. Cholesterol oxidation products were separated using split injection (100:1) onto a DB-1 fused silica gel capillary column (J&W Scientific, Folsom, CA) with an inner diameter 0.25 mm, length 15m, and film thickness 0.1 µm. Helium carrier gas flow through the column was set at 1 mL/min while nitrogen auxiliary gas flow was adjusted to 27 mL/min. Oven temperature was programmed from 180°C to 290°C at 3°C/min with a final hold of 55 min. Injection port was 250°C and detector 300°C. Peak areas and retention times were calculated and recorded by a HP 7673 Series II integrator.

Cholesterol oxide peak identification was based on retention times of known standards relative to the internal standard. The presence and identification of COPs in yolk samples were also confirmed with gas chromatography-mass spectrometry (GC-MS). Standards of 5-cholestene-3β.7α-diol (7α-hydroxycholesterol), 5-cholestene-3β, 7β-diol (7β-hydroxycholesterol), 5-cholestene-3β-ol-7-one (7-ketocholesterol), cholestan-3β,5α,6β-triol (cholestane-triol), and cholestan-5α,6α-epoxy-3β-ol (cholesterol-5α,6α-epoxide) were obtained from Steraloids, Inc. (Wilton, NH) while 5-cholestene-3β-ol (cholesterol) was obtained from Sigma Chemical Co. (St. Louis, MO). Quantification of COPs in dried egg yolk was determined using the internal standard method.

Isomers, cholesterol-5α,6α-epoxide and cholesterol-5β,6β-epoxide, were not resolved by the GC method and coeluted as one peak. Thus, our references to cholesterol-5,6-epoxides refer to combined α- and β-isomers or total cholesterol-5,6-epoxide.

Experimental design

The experiment contained a replication of control and treatments, producing a total of 8 batches of yolk: two control batches and 2 for each treatment. Control and treatment batches were prepared and spray-dried in random order. Position of storage in the oven was randomly assigned. Within each time interval, duplicate samples were analyzed for COPs in randomized sequence. Acquired data were analyzed for significant treatment effect using analysis of variance, and contrasts were used to iden-

tify statistical differences among treatments and control. For all statistical tests, significance was established at p $\leq$ 0.05.

RESULTS & DISCUSSION

Initial COP levels

No apparent differences in initial COP levels were observed for any treatments or the control. Initial mean COP levels (followed by respective standard deviations) for freshly spray-dried egg yolk with added Cu^{+2} were: 7-ketocholesterol: 6.8 µg/g (1.4), 7α-hydroxycholesterol: 4.2 µg/g (1.1), 7β-hydroxycholesterol: 3.5 µg/g (0.8), cholestane-triol: 0.9 µg/g (0.3), cholesterol-5,6-epoxide: 1.0 µg/g (0.3). Preliminary work demonstrated slightly higher initial COP levels in yolk spray-dried in the presence of added Cu^{+2} catalyst compared to those of uncatalyzed spray-dried yolk. The metal catalyst also appeared to promote 7-ketocholesterol over 7α- and 7β-hydroxycholesterol formation, whereas the opposite was observed for uncatalyzed spray-dried yolk.

Direct comparison of initial COP levels of our study with reported studies is difficult since studies differed with regard to COPs measured, food products analyzed, and various spray-drying parameters. However, our initial COP levels were reasonably consistent with published values. Our observed cholesterol-5,6-epoxide levels fell within the 0-5 ppm range of Tsai and Hudson (1985) from 9 fresh commercial dried yolk samples and compared favorably with the undetectable levels reported by Morgan and Armstrong (1987). Our epoxide levels fall below the specified 2.5 ppm detection limit of their study. An indirect heating source was common to both previous studies as well as our study. Since only epoxides were isolated in the two referenced studies, further comparison of COPs was not possible.

Nourooz-Zadeh and Appelqvist (1987) reported trace amounts of cholesterol-5,6-epoxides, and 7α- and 7β-hydroxycholesterol in fresh commercial spray-dried yolk. Cholestane-triol and 7-ketocholesterol were not detected. In analyzing newly dried commercial egg yolk, Sander et al. (1989) reported somewhat higher levels of 7β-hydroxycholesterol (51 ppm) and cholesterol-5,6-epoxides (109 ppm) than our results. Cholestane-triol, 7-ketocholesterol, and 25-hydroxycholesterol were also detected. In comparison to our results, Fontana et al. (1993) reported similar levels of 7α-hydroxycholesterol, 7β-hydroxycholesterol, 7-ketocholesterol and cholesterol-5,6-epoxides in newly spray-dried whole egg. Cholestane-triol was not detected. Unspecified spray-drying parameters and differences in product composition preclude further comparison.

Accelerated storage effect on COPs

Elevated temperature storage resulted in increased individual COP levels (Fig. 1). This observation was consistent with other investigators (Tsai and Hudson, 1985; Morgan and Armstrong, 1987). A 60°C storage temperature hastened the rate of oxidation and at the same time encouraged progression of ambient temperature oxidative mechanisms and minimized artifact forming reactions. (Frankel, 1993). Oxidative deterioration was also expedited through incorporation of Cu^{+2} into yolk before drying. The oxidized compounds involving C-7 (carbon 7 on cholesterol) were the primary oxidation products with 7-ketocholesterol predominant followed by 7β-hydroxycholesterol and 7α-hydroxycholesterol. The latter 2 compounds were present in $\approx$ a 1:1 ratio. At elevated temperatures, oxidized compounds involving C-7 were the expected primary oxidation products in dried egg. Heat stimulates scission of cholesterol 7-hydroperoxides (Fontana et al., 1993). In addition, the presence of added Cu^{+2} favors breakdown of peroxides. Likewise, cholesterol-5,6-epoxides, (formation generally dependent on peroxides, Smith, 1992), are not favored products at elevated temperatures (Fontana et al., 1993). Epoxide formation we observed seemed to confirm this. Increases in cholestane-triol, a breakdown product
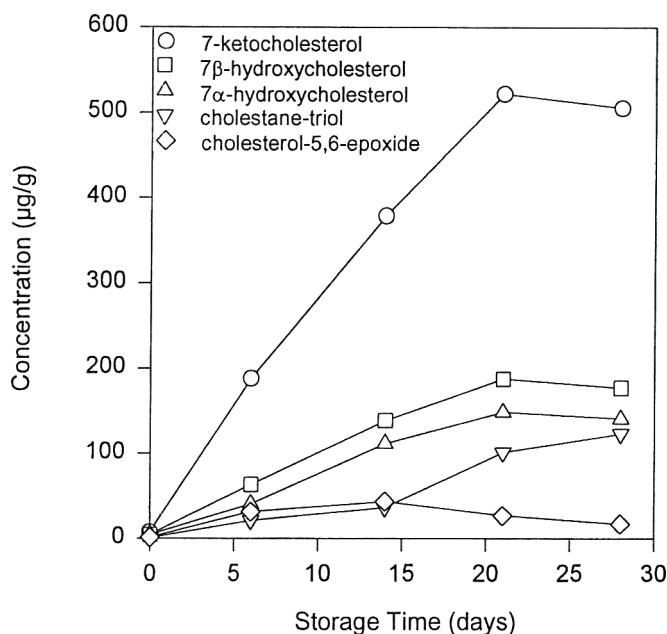
Fig. 1—Relative amounts of COPs in spray-dried egg yolk with no added antioxidant during accelerated storage (Cu^{-2} catalyzed, 60°C).

Fig. 2—Effect of antioxidants on 7-ketocholesterol formation in spray-dried egg yolk during accelerated storage (Cu^{-2} catalyzed, 60°C).

Fig. 3—Effect of antioxidants on 7α-hydroxycholesterol formation in spray-dried egg yolk during accelerated storage (Cu^{-2} catalyzed, 60°C).

of epoxides (Smith, 1992), coincided with the decrease in epoxide levels after 14 days storage (Fig. 1).

Levels of 7-ketocholesterol, 7α-hydroxycholesterol, and 7β-hydroxycholesterol reached a maximum at 22 days and then leveled off (Fig. 1). This may be associated with depletion of oxygen over time. Since samples were stored in glass vials tightly secured with Teflon-lined screw caps, oxygen levels were expected to decrease with time as a result of general lipid oxidation.

The capped storage container we used was intended to retain moisture during elevated temperature storage, allowing oxidation of normally hydrated spray-dried yolk. Two faulty sample vials discovered during the course of the study were ineffective moisture and oxygen barriers. Both resulting yolk samples had high levels of COPs compared to samples effectively secured. Unlimited availability of oxygen and the loss of moisture during elevated temperature storage may explain the increased oxidation levels of the 2 samples stored in faulty vials. Oxygen and water activity effects on oxidation have been well documented.

Antioxidants were evaluated for ability to inhibit formation of individual COPs. Treatment effects were related to the control (Fig. 2–6) for each individual COP. The tocopherol blend and BHA significantly inhibited 7-ketocholesterol (Fig. 2), 7α-hydroxycholesterol (Fig. 3), and 7β-hydroxycholesterol (Fig. 4) formation with respect to the control. Ascorbyl palmitate was more effective than the control in inhibiting formation of only 7-ketocholesterol (Fig. 2) and 7β-hydroxycholesterol (Fig. 4). No significant differences were observed between BHA and the tocopherol blend or between BHA and ascorbyl palmitate. However, the tocopherol blend was more effective than ascorbyl palmitate in preventing 7α-hydroxycholesterol and 7-ketocholesterol formation. In an aqueous model system evaluating antioxidants at 0.01% (w/w, based on total lipid), tocopherol isomers proved effective inhibitors of 7-ketocholesterol formation while BHA and several novel antioxidants exhibited no inhibitory effect relative to the control (Rankin and Pike, 1993). In our study, BHA at the same 0.01% level (w/w, based on total lipid), significantly inhibited formation of several COPs, including 7-ketocholesterol. The tocopherol blend we used, although present in greater quantity than BHA on a percentage basis (0.023% vs 0.01%), was also an effective inhibitor. It was not significantly different than BHA in preventing formation of any COPs. The same order of antioxidant effectiveness was observed
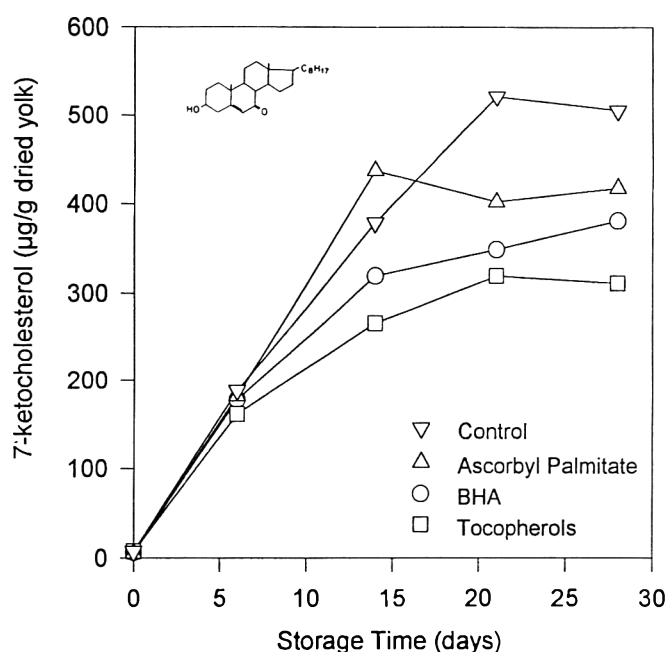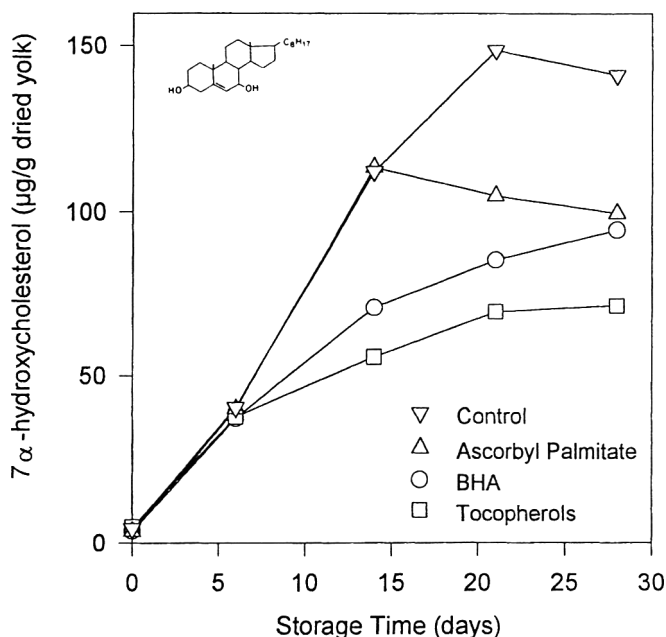
for inhibition of all oxidized products involving carbon 7 of cholesterol (Figs. 2–4). Thus, additional differences between treatments might have been obscured by experimental error.

Cholestane-triol (Fig. 5) and cholesterol-5,6-epoxide (Fig. 6) levels were not significantly inhibited by antioxidants. This has been reported in prior studies involving similar food products. Morgan and Armstrong (1987) reported BHA, BHT, and propyl gallate (67-200 ppm in yolk solids) did not inhibit cholesterol-5,6-epoxide formation during ambient temperature storage of hydrogen peroxide-treated, spray-dried egg yolk. Additionally, at antioxidant levels comparable to those of our study, α-tocopherol did not appear to inhibit cholesterol-5,6-epoxide or cholestane-triol formation in spray-dried whole egg stored at
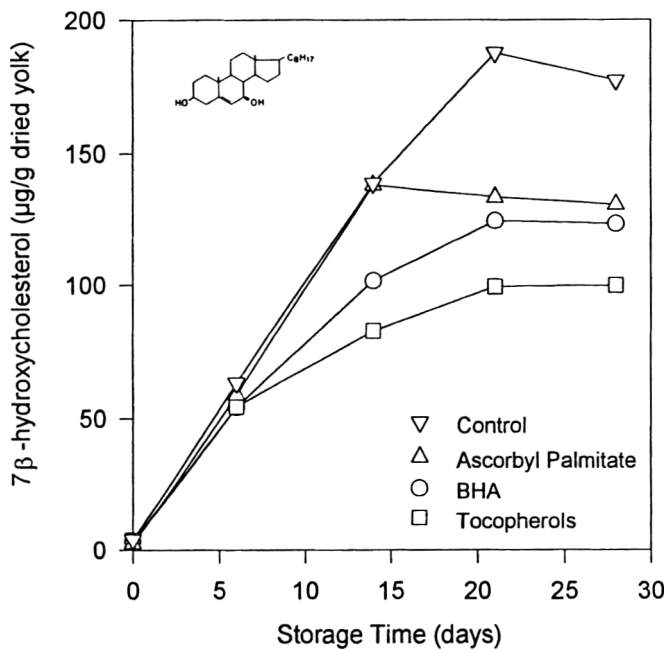
Fig. 4—Effect of antioxidants on 7β-hydroxycholesterol formation in spray-dried egg yolk during accelerated storage (Cu^{-2} catalyzed, 60°C).
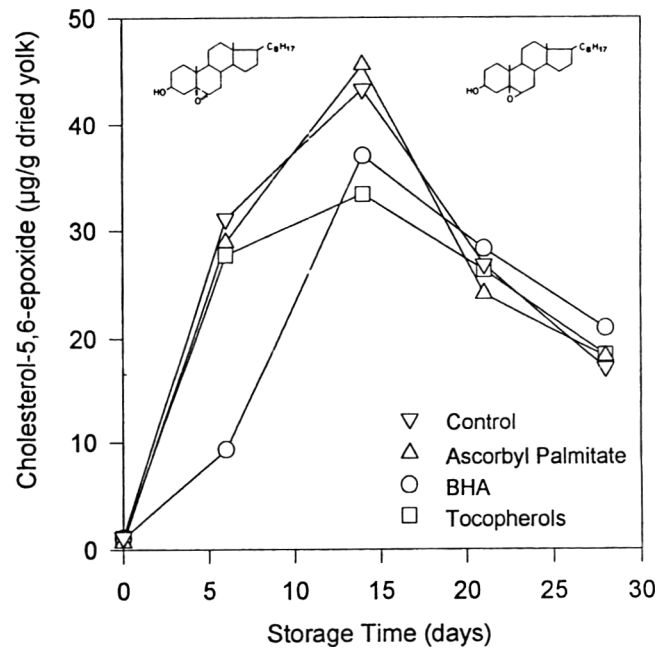
Fig. 6—Effect of antioxidants on cholesterol-5,6-epoxide formation in spray-dried egg yolk during accelerated storage (Cu^{-2} catalyzed, 60°C).
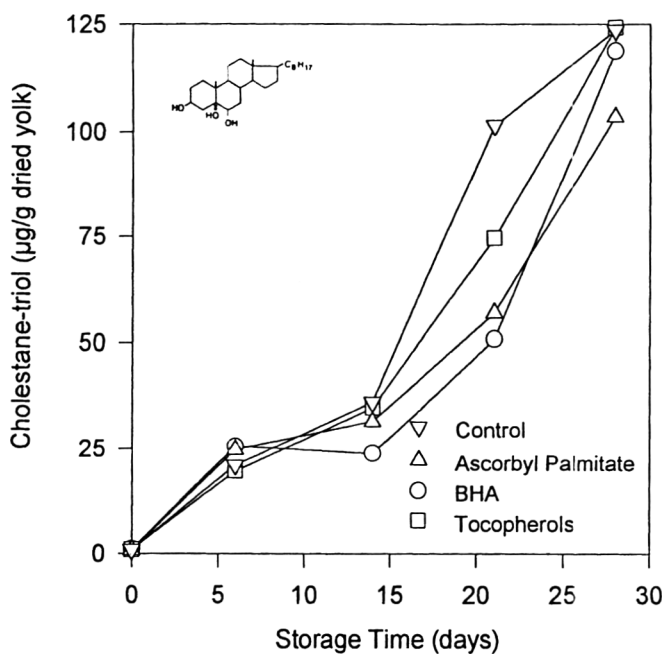
Fig. 5—Effect of antioxidants on cholestane-triol formation in spray-dried egg yolk during accelerated storage (Cu^{-2} catalyzed, 60°C).

ambient temperature exposed to light (Wahle et al., 1993). Although not statistically evaluated in their study, α-tocopherol levels > 500 µg/g dried egg provided some inhibition of both these COPs. Thus, antioxidant levels higher than those in our study might effectively inhibit cholestane-triol and cholesterol-5,6-epoxide formation in food. The presence of either of these COPs in food has potential health implications (Peng et al., 1992a, b; Morin and Peng, 1992; Morin et al., 1992). Additionally, the selective antioxidant inhibition of COPs emphasizes the need to quantitate all oxides when evaluating COP inhibition.

Although antioxidants were evaluated in a spray-dried food, the addition of Cu^{+2} and the elevated storage temperature used to accelerate the study comprised a model system. Such a system

has the advantage of providing quick results but a possible disadvantage of limited application. This study was designed to increase the applicability of results to spray-dried products stored at ambient temperature. Storage vials were capped to prevent moisture loss. A 60°C storage temperature was utilized instead of higher temperatures often used in such studies. This helped promote progression of ambient temperature oxidative mechanisms (Frankel, 1993).

Though antioxidants did not completely eradicate COPs, their formation was inhibited. The tocopherol blend, which proved effective, is not restricted by set levels and may be added in unrestricted quantities. Higher levels may more effectively inhibit COP formation.

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Cholesterol Oxidation in Baked Foods Containing Fresh and Powdered Eggs

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─────── ABSTRACT ───────

The degree of cholesterol oxidation in commercial sweet baked foods (biscuits and snacks) and in laboratory baked biscuits, all containing fresh or powdered eggs, was determined. 7-Ketocholesterol was used as index of cholesterol oxidation and detected by two analytical methods. The analysis of the biscuits showed higher levels of 7-ketocholesterol and a more marked oxidative instability of cholesterol when prepared with powdered eggs. The significant amounts of 7-ketocholesterol found in some samples of commercial biscuits were attributed to the use of powdered eggs. These data are of importance to industries using eggs in sweet baked products which are mainly consumed by children.

Key Words: eggs, cholesterol, oxidation, biscuits, snacks

INTRODUCTION

THE GROWING INTEREST in oxidation of cholesterol in foods is linked to the potential toxicity of certain components. Many cholesterol oxidation products may be cytotoxic, mutagenic, atherogenic, carcinogenic and inhibit cholesterol biosynthesis (Bishoff, 1969; Taylor et al., 1979; Smith, 1981; Parish et al., 1986; Maerker, 1987; Watanabe et al., 1988; Addis, 1990; Addis and Warner, 1991; Bösinger et al., 1993); Kandutsch et al. (1978) reported that a low level of oxysterols (less than 10^{-6} – 10^{-9}M), was biologically active.

Various analytical techniques have been developed to identify and quantify oxysterols in foods (Guardiola et al., 1994). Cholesterol oxidation products were found in milk and dairy products (Nourooz-Zadeh and Appelqvist, 1988a, b), infant formulas (Zunin et al., 1990), meat (Park and Addis, 1987; Zubillaga and Maerker, 1991; Hwang and Maerker, 1993), fish (Ohshima et al., 1993; Osada et al., 1993), fried foods (Zhang et al., 1991). Powdered eggs received particular analytical attention by many researchers (Chicoye et al., 1968; Tsai and Hudson, 1984, 1985; Naber and Biggert, 1985; Missler et al., 1985; Nourooz-Zadeh and Appelqvist, 1987; Morgan and Armstrong, 1989) because of the high cholesterol content of eggs and the considerable increased risk of oxidation by spray-drying. Powdered eggs are used as ingredients in many foods that are subjected to subsequent cooking treatments, which, in turn, may lead to further increase of cholesterol oxidation.

Wide-ranging experiments with varying analytical approaches (HPLC, HRGC, GC-MS) established that 7-ketocholesterol is a useful index of cholesterol oxidation (Caboni et al., 1991; Rankin and Pike, 1993). It was observed that 7-ketocholesterol forms early in model systems, always resulting in an amount higher or similar to that of the main oxysterols (Nourooz-Zadeh and Appelqvist, 1988a). Even if 7-ketocholesterol tends to break down slowly analytical methods employed for its determination are very sensitive and thus are particularly reliable for detection of cholesterol oxidation.

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Our research groups separately developed two different analytical methods to determine 7-ketocholesterol and then co-operated by comparing results. Previous studies of very small quantities of this subtance had yielded conflicting results. The two methods were widely and successfully tested for determination of 7-ketocholesterol in egg powders (Caboni et al., 1991). In our study they were both employed to determine the degree of cholesterol oxidation of industrial baked products, such as biscuits and sweet snacks, prepared with fresh or powdered eggs. In such products high temperature baking and long storage periods contribute towards increasing cholesterol oxidation. However, the presence of other cholesterol-containing ingredients, such as butter and milk, prevents relating the measured levels of 7-ketocholesterol solely to oxidation of egg cholesterol. Our objective was to study more accurately the incidence of the type of eggs on the degree of cholesterol oxidation in a baked food and to follow the development of the oxidation process when a baked product contained fresh or powdered eggs. Two biscuit types were prepared in our laboratory using only flour, fresh or powdered eggs and sugar. This experimental approach enabled us to investigate the influence of baking on cholesterol level as well as the evolution of oxidation over storage life.

MATERIALS & METHODS

Samples

Two groups of commercial samples were purchased in local stores and analyzed: 10 biscuits and 10 sweet snacks. Two kinds of biscuits, containing either four fresh or 40g powdered eggs, 40g water, 200g flour, and 200g sugar in the dough, were then baked in laboratory at 185°C for 20 min. The two kinds of laboratory baked biscuits, prepared with fresh or powdered eggs, were divided into two parts: one part was packed and sealed, while the other was ground and immediately analyzed. A 5-g sample of each type biscuit was then extracted. The packed biscuits were stored at room temperature (≈23°C) and subjected to extraction after a 35-day storage. Samples of fresh and powdered eggs were also analyzed.

Reagents

7-Ketocholesterol and cholesterol were purchased from SIGMA Chemica (St. Louis, MO); 7-ketopregnenolone, the internal standard for method A, was supplied by Steraloids (Wilton, NH).

Methods

Extraction of lipids. The method employed was that of Folch et al. (1957). Clean-up and HPLC analysis of lipids extracted from the commercial samples were performed by methods A and B and those extracted from the laboratory samples by method B alone

Method A. Cholesterol oxides were enriched by solid-phase extraction (SPE): 1.5 µg 7-ketopregnenolone were added to 50–60 mg of precisely weighed extracted lipids and dissolved in 0.5 mL of 2% 2-propanol in n-heptane. This solution was applied to a 500mg Bond Elut Florisil cartridge (Varian, Harbor City, CA) prewashed with n-heptane. Triacylglycerols and most of the cholesterol were removed with 4 mL of 2% 2-propanol in n-heptane, and any retained cholesterol oxides were then eluted in 5 mL acetone. This solvent was evaporated under nitrogen flow (T≤30°C). HPLC was run with a 30 cm × 3.9 mm i.d. Waters µPorasil column (Milford, MA). The mobile phase was 93:7 n-hexane/2-propanol (v/v) with a 1.0 mL/min flow (Park and Addis, 1985). The Perkin Elmer

LC system (Norwalk, CT) consisted of a Series 400 liquid chromatograph (loop 20 μL) connected to an LC 90 liquid spectrophotometric detector (λ = 233 nm) and a CC-12 computing integrator. Quantification was by the internal standard method, with multiple level calibration (R^2 = 0.9984).

Method B. Cholesterol oxides were enriched by SPE: about 100 mg of lipids, exactly weighed, were dissolved in 0.5 mL n-hexane and applied to a 200 mg Bond Elut Silica cartridge (Varian, Analitichem International, Harbor City, CA) which was prewashed with n-hexane. Elution was carried out with 3 mL of n-hexane:diethylether (8:2), 4 mL of n-hexane:ether (1:1) and 3 mL of methanol (Bortolomeazzi et al., 1990). The two last fractions were mixed and solvents were eliminated by evaporation under nitrogen flow (T≤40°C); samples were then redissolved in 3 mL acetonitrile. The HPLC system included a Knauer pump (Berlin, Germany) (loop 20 μL) connected to a Knauer variable UV-wavelength detector through a Knauer λ-selector; wavelengths used for detection were: 7-ketocholesterol 245 nm and cholesterol 210 nm. HPLC was run with a Spherisorb Phase Sep C6 column (15 cm × 4.6 mm i.d., 5 μm) (Deeside, UK). The mobile phase was 80:20 acetonitrile/water (v/v) with 0.8 mL/min flow. The integrator was a Spectra Physics 4290 (San Jose, CA). Quantification of 7-ketocholesterol and cholesterol was by the external standard method (R^2 = 0.9996 for 7-ketocholesterol).

7-Ketocholesterol identification was performed by GC-MS of the trimethylsilyl derivatives (Sweeley et al., 1963) after cold saponification (Sander et al., 1989). Analysis was run on a Carlo Erba QMD 1000 system (Rodano, Milano, Italy) equipped with an SE 52 column (25 × 0.25 mm i.d., 0.10 μm film thickness) (Chrompack, Milano, Italy). Column temperature rose from 60 to 260°C at 25°C/min and then to 310°C at 2°C/min; oven temperature was 60°C at on-column cold injection (Galli et al., 1979). Transfer line was 250°C and the source was 200°C. Ionization energy was 70 eV. Mass spectra were recorded within 40-600 mass range (m/z); scan speed was 0.9 m/z sec^{-1}.

RESULTS & DISCUSSION

FOR BOTH METHODS the minimum quantifiable amount was 3 × 10^{-9}g for 7-ketocholesterol. Three individual measurements/sample/method were performed: standard deviations (ppm in lipids) and standard errors were σ = 3.1 × 10^{-1} and SE = 1.8 × 10^{-1} for method A and σ = 3.0 × 10^{-1} and SE = 1.7 × 10^{-1} for method B.

7-Ketocholesterol content in commercial biscuits

7-Ketocholesterol amounts (ppm in lipids) detected by both analytical methods were compared (Table 1); also included were the amount of extracted lipids and the cholesterol determined with method B. Reporting total lipid contents is necessary to avoid incorrect evaluations as to amounts of 7-ketocholesterol in the samples. The values of 7-ketocholesterol recorded with the two methods were in good agreement, as shown by the high correlation coefficient calculated for the samples 1–9 (r^2 = 0.996); sample 10 was excluded as it was far higher than all others. Had the two methods been identical, the points would have plotted a straight line with slope 0 and intercept 1. The B = 0.829A + 0.863 linear equation in this case did not indicate this. The large number of ingredients in the analyzed products sometimes made oxysterols purification difficult, thus leading to slight differences between detected amounts of 7-ketocholesterol.

Possibly the first four samples, whose ingredient compositions were not fully described, were not prepared with powdered eggs, whose usual content of 7-ketocholesterol is markedly higher than the measured amounts. Two of the samples contained powdered skimmed milk, but its addition to overall fatty substance was negligible. Samples 2 and 5 contained butter, in a marked amount, and eggs, as confirmed by the high cholesterol amounts shown. The processing conditions of these samples would not induce a marked development of oxidation. Note that sample 1 was the only one in which "fresh eggs" were specifically listed among ingredients. Note also that sample 3 was a batch of biscuits for infants: safeguarding the integrity of such products is even more important when considering the targeted consumers.

Samples 8, 9, and 10 showed the highest amounts of 7-ketocholesterol. Sample 8 listed whole milk powder among its ingredients; in samples 9 and 10, eggs were listed as the third ingredient and in sample 10 they accounted for 15.7% of total ingredients. The biscuits of this latter sample had a very low fatty-substance content (Table 1), having neither vegetable nor animal fats in their dough. Thus, apart from the lipid fraction in the flour, the extracted fatty substance was mostly attributable to the eggs. Since powdered eggs have a 7-ketocholesterol amount comparable to that found in our samples (Caboni et al., 1991), we postulated that these biscuits were made with them. Yet, although the 7-ketocholesterol amounts were high in relation to extracted fatty substance, the lipid fraction was small enough so that the cholesterol oxides were no cause for alarm if the reference was 100g of biscuit.

The greater amounts of 7-ketocholesterol detected in samples 5, 6 and 7 as compared to the first sample group were likely related to differing baking techniques.

7-Ketocholesterol content in commercial sweet snacks

The 7-ketocholesterol amounts (ppm in lipids) found with the two methods in sweet snacks were compared (Table 2). The extracted fatty substance and cholesterol amounts per sample were also included. The correlation coefficient (r^2 = 0.934) showed that results of the two methods were in good agreement. The slope 1 and intercept 0 postulate could be accepted in this case and the linear regression equation was B = 0.870A + 0.223, further confirmation that either of the methods could be used with equally satisfactory results. Note also that there was a degree of uniformity of recorded values, which were lower ($\bar{x}_A$ = 3.17, σ_A = 1.44; $\bar{x}_B$ = 2.98, σ_B = 1.35) than those for the biscuits ($\bar{x}_A$ = 6.88, σ_A = 8.04; $\bar{x}_B$ = 6.97, σ_B = 7.80). This may be attributed to the higher moisture content of these snacks compared with the biscuits: Caboni et al. (1992) reported that a model-system analysis indicated that water had an inhibitory effect on the complex reactions in cholesterol oxidation. Furthermore, the larger size of snacks as compared to biscuits should reduce the impact of processing technologies because less surface per unit volume would be exposed to oxygen.

Of the samples, five formed a group marked by 7-ketocholesterol values ranging from 1.6 to 2.9 ppm on the extracted lipids; three had values <2.0 ppm. Among their ingredients there were neither whole milk powder, nor powdered eggs. Obviously, production conditions did not cause a marked oxidation of cholesterol. The other five samples listed either powdered or concentrated milk or powdered eggs among their ingredients.

7-Ketocholesterol content in laboratory baked biscuits

The 7-ketocholesterol values recorded in the laboratory biscuit samples immediately after baking (F_0 and P_0) and after 35-day storage (F_{35} and P_{35}) as well as in fresh and powdered eggs were compared (Fig. 1). All data represent to the mean of two replicates. The fresh eggs showed only trace amounts of 7-ketocholesterol (≤0.5 ppm in lipids). The same low values were also found in the fresh eggs analyzed after 8 mo frozen storage, as also reported by Nourooz-Zadeh and Appelqvist (1987). This processing technique did not affect the oxysterols concentration and, hence, the use of frozen instead of fresh eggs could be considered as having no effect on cholesterol oxidation.

The 7-ketocholesterol amount detected in the powdered egg used to prepare the biscuits was 4.7 ppm in lipids (whose content was 54% by weight), i.e. lower than reported (Sander et al., 1989; Caboni et al., 1991). This also has to be considered in relation to dehydrating techniques and storage time and modes used for the powdered eggs. However, the 7-ketocholesterol value was much higher than in fresh eggs and also higher than the amount found after baking biscuits made with them. The 7-ketocholesterol amount recorded in F_0 and P_0 biscuits was 3.9 and 13.0 ppm (in extracted lipids), respectively, and both types

Table 1—7-ketocholesterol amount in egg containing commercial biscuits

Sample	Extracted lipids (g/100g)	Other cholesterol containing ingredients	Cholesterol (mg/100g)	7-ketocholesterol (ppm in lipids) Method A	7-ketocholesterol (ppm in lipids) Method B
1	17.4	powdered skim milk	30	1.1	2.5
2	20.8	butter (20%), milk	150	1.2	1.8
3	9.7	butter, powdered skim milk	30	2.2	2.7
4	27.6	none	60	2.2	2.5
5	13.8	butter (20%)	100	3.5	3.7
6	21.0	butter	80	3.8	3.6
7	20.4	butter	140	4.4	4.3
8	13.4	butter, whole powdered milk	80	11.4	10.4
9	4.2	none	130	12.2	11.1
10	3.7	none	140	26.8	27.1

Table 2—7-ketocholesterol amount in egg containing commercial sweek-snacks

Sample	Extracted lipids (g/100g)	Other cholesterol containing ingredients	Cholesterol (mg/100g)	7-ketocholesterol (ppm in lipids) Method A	7-ketocholesterol (ppm in lipids) Method B
11	24.4	Yogurth, fresh milk	110	1.6	1.8
12	29.2	butter, fresh milk	120	1.8	2.0
13	30.9	butter	120	1.9	1.5
14	29.4	butter, fresh milk	110	1.9	2.2
15	20.7	butter	130	2.9	2.4
16	15.3	none	120	3.3	2.5
17	17.8	powdered milk	160	3.5	3.8
18	19.2	butter, concentrated milk	150	4.4	4.7
19	23.0	skim yogurth, whole powdered milk	170	4.4	3.3
20	22.4	butter, powdered skim milk	240	6.0	5.6
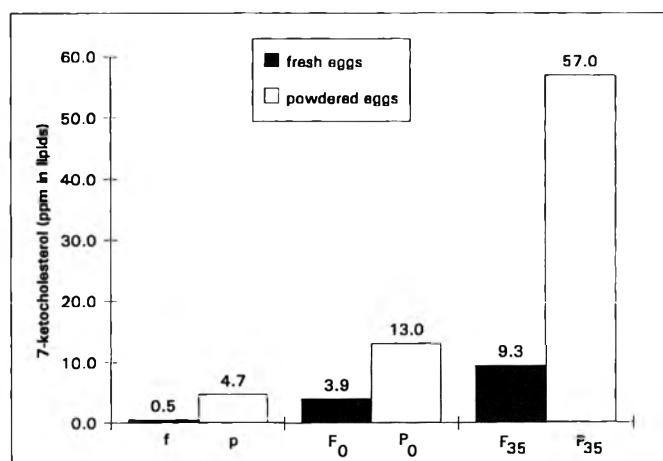
Fig. 1—Laboratory baked biscuits: 7-ketocholesterol content of fresh (f) and powdered (p) eggs employed and of samples immediately after baking (F_0 and P_0) and after a 35 day storage (F_{35} and P_{35}).

showed 7.4% extracted fat. These samples showed a more extensive cholesterol oxidation after baking than before. Such finding was reported for other foods by Park and Addis (1986), Nourooz-Zadeh and Appelqvist (1988b), Yan and White (1990), and Pie et al. (1991).

The 7-ketocholesterol amount determined after 35-day storage at room temperature (20°C) in a sealed package was 9.3 in F_{35} and 57.0 in P_{35} lab-baked samples. The increase of cholesterol oxidation products during storage was reported in other food products such as powdered milk (Nourooz-Zadeh and Appelqvist, 1988a), butter (Nourooz-Zadeh and Appelqvist, 1988b) and dehydrated eggs (Nourooz-Zadeh and Appelqvist, 1987). Our storage period was shorter than the industry standard because of the lack of adequate packaging over a longer period of time.

Our data indicate that powdered eggs, apart from being rich in 7-ketocholesterol *per se*, impart a degree of oxidative instability of cholesterol to foods. Oxidation of cholesterol in such products occurs much faster than in similar products made with fresh eggs. 7-ketocholesterol values recorded from biscuits and

snacks showed that they had variable amounts of oxysterols. The 7-ketocholesterol of some types of biscuits could be be attributed to the powdered eggs. The analysis of laboratory baked biscuits also showed a more marked oxidative instability of cholesterol in baked foods containing powdered eggs.

The data are important for food technologists, since industries utilize food ingredients that may be high-risk with regard to oxidation. Particular attention should be given to products prepared with such ingredients and mainly consumed by children. Research is needed on developing processing conditions of ingredients and final products as well as storage conditions that generate a minimum degree of cholesterol oxidation.

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Ms. received 12/22/94; revised 3/14/95; accepted 4/11/95.

Gel Forming Characteristics of Frozen Surimi from Chum Salmon in the Presence of Protease Inhibitors

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—————————— ABSTRACT ——————————

When salted surimi paste of chum salmon was incubated at 20–60°C, a marked loss of the breaking strength of heat-induced gel occurred simultaneously with breakdown of myosin heavy chain, but this was effectively suppressed by addition of cysteine protease inhibitors or bovine plasma powder. In the presence of protease inhibitor, the surimi gels were formed at relatively low temperatures showing highest gel strength at incubations of 50 and 60°C. Chum salmon surimi showed no evidence of suwari and no myosin heavy chain cross-linking.

Key Words: surimi, chum salmon, gel formation, protease inhibitors

INTRODUCTION

GEL FORMING ABILITY of frozen surimi is the most important functional requirement imposing good quality on surimi-based products. The textural profile of the heat-induced gel varies with the heating schedule of salt-ground meat (Katoh et al., 1984; Numakura et al., 1990). That is, when salted surimi paste of walleye pollock is incubated below 40°C, and subsequently heated at 90°C, a highly elastic gel is produced (Migita and Okada, 1952; Lanier et al., 1981). This is termed suwari (setting). Studies have reported that the gel formation of salted surimi paste of walleye pollock at low temperature closely correlated with a covalent cross-linking reaction of myosin heavy chain (Numakura et al., 1985, 1990; Saeki et al., 1992; Kamath et al., 1992). However, a marked loss of elasticity of heat-induced gels occurs when the salted surimi paste is incubated at 50–60°C. The gel degradation induced by heating of fish meat gel is called modori and impairs the textural quality of surimi-based products (Okamura, 1961; Shimizu et al., 1981; Makinodan et al., 1985). These characteristics of gel formation of fish meats are important in the manufacture of surimi-based products.

Chum salmon is an important fish resource in the northern Pacific. Some attempts have been made to manufacture frozen surimi from fall spawning chum salmon because of the abundant supply and low value. Chum salmon muscle, especially in the spawning stages, contains cathepsins and alkaline proteases with high proteolytic activity (Konagaya, 1985; Nomata et al., 1985; Yamashita and Konagaya, 1990). Nishimoto et al. (1988) reported that suwari was not observed in heat-induced gelation of chum salmon meat paste at 25°C and 40°C. However, there has been no report of an investigation of the participation of proteases in the gel forming characteristics of this species, especially in suwari and modori.

Our objectives were to elucidate the gel forming characteristics of chum salmon meat paste and to investigate optimum conditions for its heat-induced gelation. In addition, we studied the effects of protease inhibitors and commercial bovine plasma powder on heat-induced gel formation in suwari and modori.

MATERIALS & METHODS

Frozen surimi

Frozen surimi of the fall spawning population of chum salmon was produced experimentally according to the procedure for making walleye

pollock surimi (Toyoda et al., 1992). Raw fish used were of good quality within one day postmortem and were processed into surimi within 8 hr. The composition of this surimi was as follows: moisture, 73.8% (W/W); crude protein, 19.1%; myofibrillar protein, 14.9%; lipids, 0.5%; sucrose, 6.0%; and polyphosphate, 0.25%. The pH was 6.9 and myofibrillar Ca-ATPase activity (Katoh et al., 1977) of the surimi was 0.296 μmol Pi/min·mg of protein. Frozen surimi blocks (10 kg) were cut into about 300-g portions, vacuum-packed in oxygen-impermeable bags, and stored at −30°C until used.

Preparation of heat-induced gels

Frozen surimi was cut into small cubes (about 1 cm). Partially thawed surimi cubes were placed in a food cutter (model MK-K74; Matsushita Electric Industry Co., Ltd., Osaka, Japan). After chopping for 1 min, cold distilled water was added for adjusting protein concentration to 180 mg/g and chopping was continued for 30 sec. The chopped surimi was ground with 3% (w/w) NaCl for 4 min at 4°C. Salted surimi paste thus obtained was stuffed into a polyvinylidene casing (diam, 22 mm; length, 200 mm). Temperature of surimi paste was maintained at <8°C. Two sets of heat-induced gels (1-step heating gels and 2-step heating gels) were prepared by the following methods: the 1-step heating gels were prepared by incubating the salted surimi paste in a water-bath adjusted to 10, 20, 30, 40, 50, 60, 80, and 90°C for various lengths of time. The 2-step heating gels were also prepared from the 1-step heating gels, followed by further heating at 90°C for 30 min. Heated gels were immediately chilled to <5°C in ice water before storing at 5°C.

Gels with protease inhibitors or bovine plasma powder

Leupeptin, pepstatin A, and N-[N-(L-3-trans-carboxyoxiran-2-carbonyl)-L-leucyl]agmatine (E-64) were purchased from the Peptide Institute, Inc. (Osaka, Japan). Soybean trypsin inhibitor and all other chemicals (reagent grade) were obtained from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Protease inhibitors were dissolved in cold water and added to the chopped surimi. After adjusting the protein concentration to 180 mg/g, surimi containing different concentrations of protease inhibitor was ground with 3% (w/w) NaCl for 4 min, incubated at 30°C for 4 hr, and then heated at 90°C for 30 min.

Dried beef plasma powder was obtained from Taiyo Chemicals Co., Ltd. (Mie, Japan). The partially thawed surimi was ground with 3% NaCl and bovine plasma powder (0, 5, 10, and 20 mg/g of salted surimi paste) for 4 min, and incubated at 30°C for 0–3 hr, followed by heating at 90°C for 30 min.

Breaking strength of heat-induced gels

The heat-induced gels were tempered for 2 hr to ambient room temperature (≈22°C) prior to analysis. Six cylinder-shaped samples (25 mm long) were cut from each gel. The breaking strength of the gels was measured on a rheometer (model J-2012; San Kagaku Co., Ltd., Tokyo, Japan) equipped with a cylindrical plunger (diam, 5 mm; depression speed, 32 mm/min) as the load value (g) at the breaking point.

Protein subunit composition of salted surimi paste and heat-induced gels

Each 100 mg of surimi paste and heat-induced gel was homogenized with 5 mL of 20 mM Tris-HCl (pH 8.0) containing 2% sodium dodecylsulfate (SDS), 8M urea and 2% β-mercaptoethanol, and heated in boiling water for 2 min, followed by stirring for 22 hr at room temperature (Numakura et al., 1985). All samples were completely dissolved in the SDS buffer. Protein concentration was adjusted to 1.0 μg/μL. Protein was determined by the biuret method (Gornall et al., 1949) after
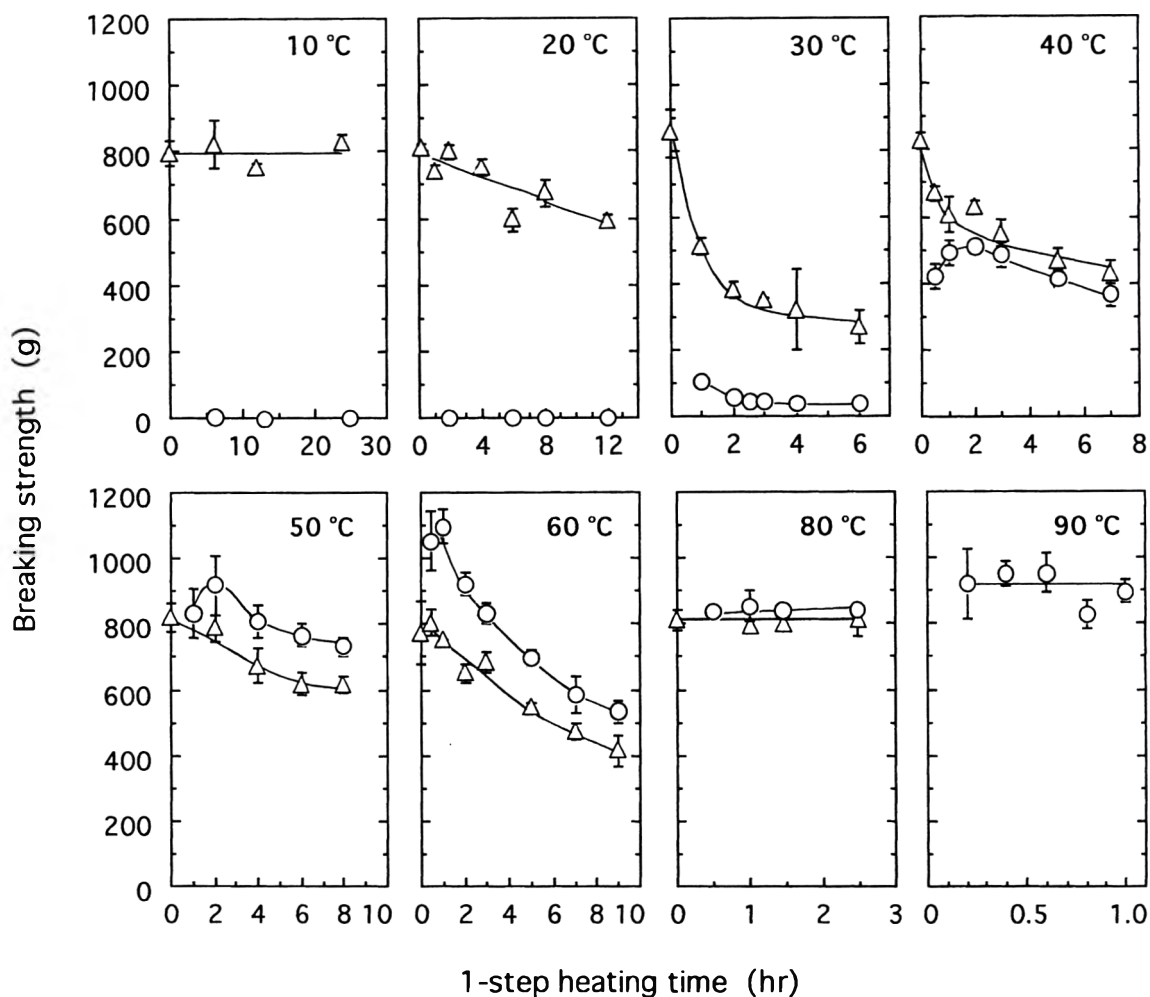
Fig. 1—Gel formation of fall spawning chum salmon surimi. The breaking strength of 1-step heating gel (o) and 2-step heating gel (△) was plotted against the step heating time of salted surimi paste at 10–90°C. Each point is the mean of six determinations. Vertical bars represent ±standard deviation.

the protein was precipitated with 7.5% (at final concentration) trichloroacetic acid to remove β-mercaptoethanol and redissolved in 1N NaOH. The solubilized proteins were subjected to SDS-polyacrylamide gel electrophoresis (Weber and Osborn, 1969) using a polyacrylamide disk gel. An Atto Disk Gel System, (model SJ-1060D; gel column size: diameter, 6mm; length, 65mm; Atto Corporation, Tokyo, Japan) was used. The disk gels were 5.0% (w/v) acrylamide and 0.14% (w/v) bis-acrylamide containing 0.1% SDS. Protein applied was 20 μL/disk gel and electrophoresis was performed at a constant current of 8 mA/disk gel using the running buffer of 0.1M sodium phosphate at pH 7.2 containing 0.1% SDS. The protein subunits on the gel rod were stained for 12 hr with 0.25% Coomassie brilliant blue R in 45% methanol and 9% acetic acid. The stained gels were destained for 24 hr with 30% methanol and 7.5% acetic acid and for 24 hr with 7.5% acetic acid (≈35°C). The content of protein components was determined by measuring the relative stain intensity on the gel rod with a densitometer (model CS-910; Shimadzu, Kyoto, Japan).

RESULTS & DISCUSSION

Changes in breaking strength and myofibrillar protein subunit

The breaking strength of heat-induced gels was measured and plotted against the 1-step heating time (Fig. 1). The salted surimi paste incubated at 10°C and 20°C did not form a gel. The 1-step heating gel was slightly formed at 30°C and more rapidly and extensively formed above 40°C. Its breaking strength increased with the rise of 1-step heating temperature and reached a maximum at 60°C. However, in the range 40–60°C, prolonged incubation caused a marked decrease in breaking strength.

Two-step heating gels were produced regardless of temperature of 1-step heating, but the breaking strength decreased with duration of 1-step heating time in the range 20–60°C. The notable decrease occurred, in particular, at 30°C and 60°C. At 10°C, 80°C, and 90°C, on the contrary, no decrease occurred in the breaking strength of both heat-induced gels. These temperature dependent decreases in breaking strength may indicate the participation of proteolysis of surimi protein. Thus, the change in myofibrillar protein components during 1-step heating was analyzed by SDS-polyacrylamide gel electrophoresis (Fig. 2).

At 20–60°C, a marked decrease in myosin heavy chain was observed with a concomitant increase in new components migrating between myosin heavy chain and actin (mainly 150 kDa and 90 kDa components). However, the other protein components including actin hardly changed. On the contrary, the protein composition scarcely changed during incubation at 10°C, 80°C, and 90°C. It is therefore obvious that the textural degradation of the 1-step heating gel at 30–60°C was attributable to the marked decrease in myosin heavy chain. No more change in myofibrillar protein components was observed when the 1-step heating gels were further heated at 90°C for 30 min in any experiment. Results indicate that the textural degradation of the 2-step heating gel was also related to changes in myofibrillar protein occurring in the 1-step heating at 20–60°C.

Suppression of gel degradation by protease inhibitors

We assumed that the gel degradation (Fig. 1) was caused by proteolysis of myosin heavy chain. In order to clarify this, we
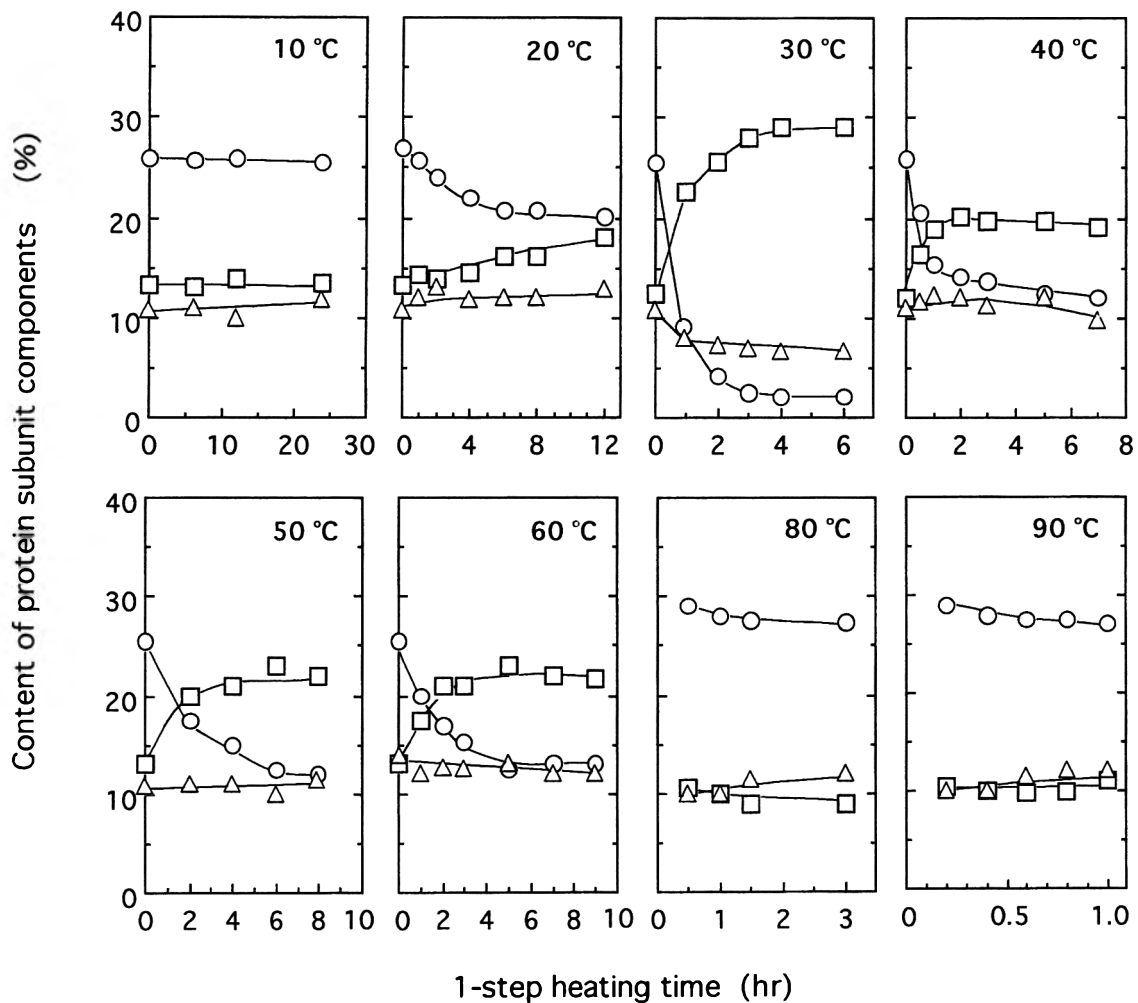
Fig. 2—Changes in protein subunit components of salt-ground meat during 1-step heating. (o) Myosin heavy chain; (△) components larger than myosin heavy chain; (□) components between myosin heavy chain and actin. Actin and components smaller than actin are not shown here because of virtually no changes in all experiments.

investigated the effects of various protease inhibitors on degradations of heat-induced gels and of myosin heavy chain. That is, the salted surimi paste containing different concentrations of E-64, leupeptin, pepstatin A, or soybean trypsin inhibitor was incubated at 30°C for 4 hr and further heated at 90°C for 30 min. The breaking strength of the 2-step heating gels thus obtained and the remaining myosin heavy chain were plotted against concentration of the protease inhibitors (Fig. 3).

The addition of E-64 or leupeptin (cysteine protease inhibitors) resulted in a marked increase in breaking strength of 2-step heating gels, showing strong suppression of degradation. The increase reached a plateau showing the maximum value >100 μg/g. Moreover, a simultaneous increase in remaining myosin heavy chains was also observed. On the contrary, no effect was observed on addition of up to 400 μg/g of pepstatin A (aspartic protease inhibitor) and 2,000 μg/g of soybean trypsin inhibitor (serine protease inhibitor). These results indicate that a cysteine protease(s) would be involved in the textural degradation of the 2-step 30°C/90°C gel and suggest the participation of cathepsin L as the most probable protease in thermal gel degradation. Yamashita and Konagaya (1990, 1991) have also reported that cathepsin L was the enzyme most likely responsible for extensive softening of muscle of chum salmon caught during the spawning season.

Gel formation in presence of E-64

Effect of E-64 at 100 μg/g on gel formation of salted surimi paste was further investigated over a range of 20–60°C. The

decrease of breaking strength of both heat-induced gels (Fig. 1) was effectively suppressed by addition of E-64 (Fig. 4A). In the presence of E-64, a strong 1-step heating gel was rapidly formed at 40°C and above, and its breaking strength reached maximum within 1 hr and remained constant thereafter. On the contrary, the strong 1-step heating gel was not produced at 20°C or 30°C although inhibitor was present.

As shown (Fig. 4B), the marked decrease in myosin heavy chain and the formation of low-molecular-weight components between myosin heavy chain and actin was also depressed by addition of E-64 at all temperatures. It is noteworthy that, even in the presence of E-64, the formation of cross-linked myosin heavy chain (because of no decrease in myosin heavy chain) and of setting gel did not occur under any conditions. These findings indicate that proteolysis was not a cause of "no setting" response.

We questioned the cause of the marked decrease in breaking strength of 2-step 30°C/90°C and 60°C/90°C gels. In the case of 2-step 60°C/90°C gel, the salted surimi paste formed a gel rapidly at 60°C (within about 30 min). As incubation time increased, the gel degraded due to proteolysis (modori). In 2-step 30°C/90°C gel, the proteolytic degradation rapidly proceeded at 30°C (Fig. 2) and no subsequent gel formation occurred at 90°C because the myosin had already degraded.

As shown (Fig. 4A), the breaking strength of the 1-step heating gel was reinforced as the 1-step heating time and temperature increased. It reached the maximum ≈1,100g within 1 hr at 60°C, whereas the 2-step heating gel showed a constant breaking strength at 900g, regardless of heating sequence. Compared with

Concentration of protease inhibitor (μg/g)
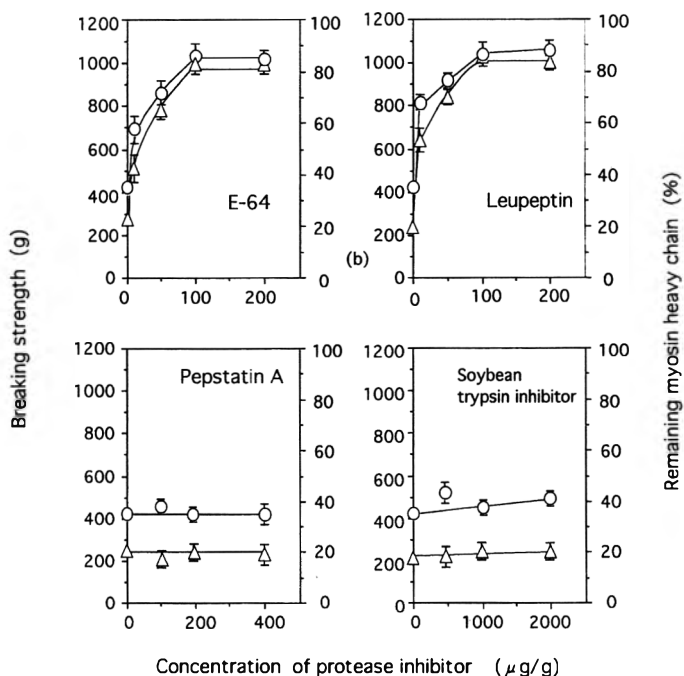
Fig. 3—Effect of chemical protease inhibitors on breaking strength of 2-step heating gel and change in myosin heavy chain content during the gel preparation. The breaking strength (o) of 2-step heating gels and the remaining myosin heavy chain (△) were measured and plotted against concentrations of protease inhibitors. Each point is the mean of six determinations. Vertical bars represent ± standard deviation.

the breaking strength of 1-step and 2-step heating gels, the 1-step heating gel once formed at 50°C and 60°C was converted to the 2-step heating gel accompanied with a 20% decrease in

breaking strength. These data indicate that salmon surimi paste formed the gel showing highest breaking strength at relatively low temperatures (50–60°C) under the condition in which the modori was completely inhibited. On the contrary, the 2-step heating gel was stronger than the 1-step heating gel at 20°C and 30°C. Such increased breaking strength with the 2-step heating was not regarded as a suwari effect. This was probably because the breaking strength of the 2-step heating gel was independent of the 1-step heating time and temperature, and it was also equal to that of the 1-step heating gel at 90°C.

Results in the presence of E-64 demonstrated the substantial improvement of gel-forming characteristics of chum salmon surimi. Studies have reported that the covalent cross-linking reaction of myosin heavy chain closely participates in the heat-induced gelation of walleye pollock (Numakura et al., 1985; Saeki et al., 1992), threadfin bream (Lee et al., 1990a) and hoki (Lee et al., 1990b). Their salted surimi pastes set into a gel readily at <30°C, and resulted in a stronger 2-step heating gel. Apparently, the gel-forming characteristics of salted surimi paste of chum salmon was completely different from that of these fish species. Based on our results, we have come to the conclusion that 1-step heating >80°C is the best method to produce a strong gel using chum salmon surimi without protease inhibitor.

Depression of gel degradation by bovine plasma powder

Attempts have been made to search for foodstuffs containing protease inhibitors for improving the texture of surimi-based products (Nagahisa et al., 1981; Toyohara et al., 1990; 1992; Kinoshita et al., 1991). Reports have suggested that bovine plasma hydrolysate was an effective enzyme inhibitor for menhaden and Pacific whiting surimi (Hamann et al., 1990; Morrissey et al., 1993; Park et al., 1994). As shown (Fig. 5), the textural degradation of 2-step heating gels accompanied by the

1-step heating time (hr)
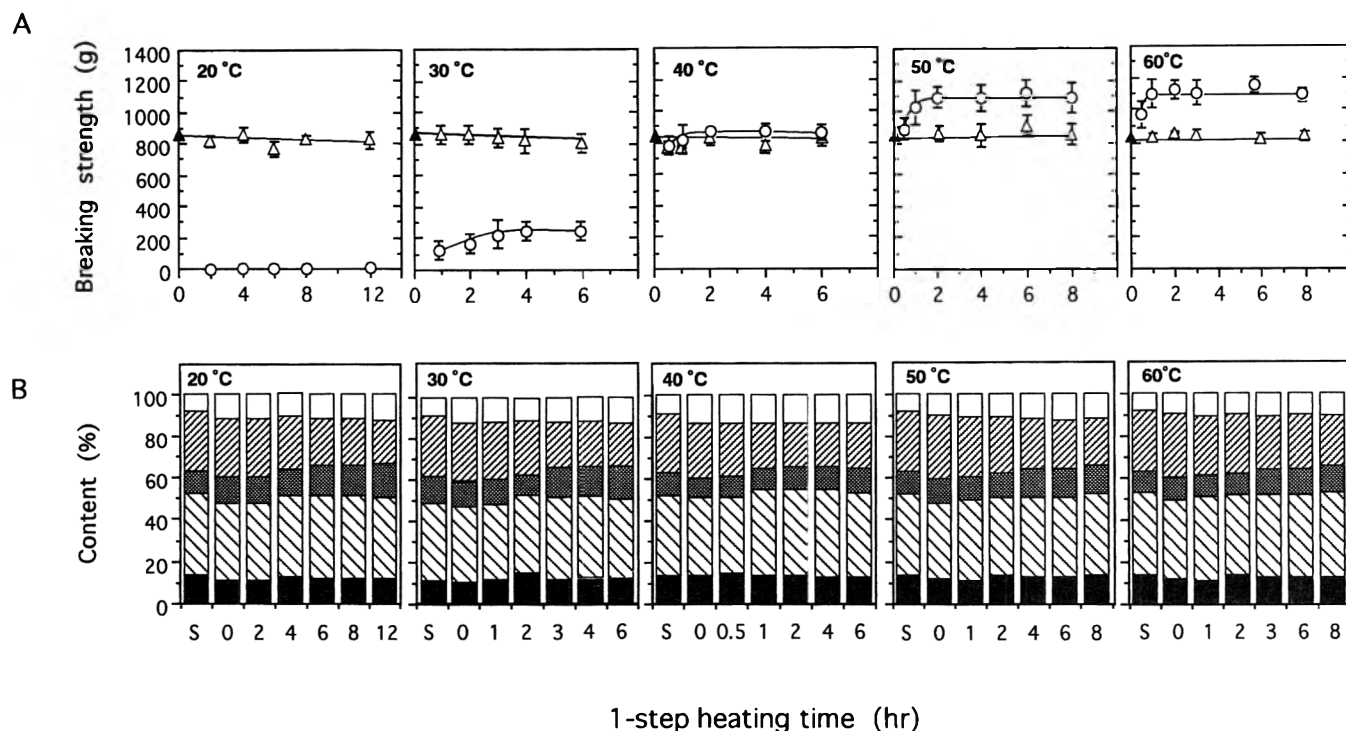
Fig. 4—Changes in breaking strength and protein subunit components of salted surimi paste containing E-64 during heat-induced gelation. (A) the breaking strength of 1-step heating gel (o) and 2-step heating gel (△) prepared from salted surimi paste containing 100 µg/g of E-64. ▲, The data of 1-step heating gel at 90°C. Each point is the mean of six determinations. Vertical bars represent ± standard deviation (n = 6). (B) column represents protein composition of salt-ground meat (S), 1-step heating gel at 90°C (0), and 2-step heating gel prepared by first-step heating at 20–60°C. □, Components larger than myosin heavy chain; ▨, myosin heavy chain; ■, components between myosin heavy chain and actin; ▨, actin and tropomyosin containing some cytoplasmic proteins; ■, components smaller than tropomyosin.
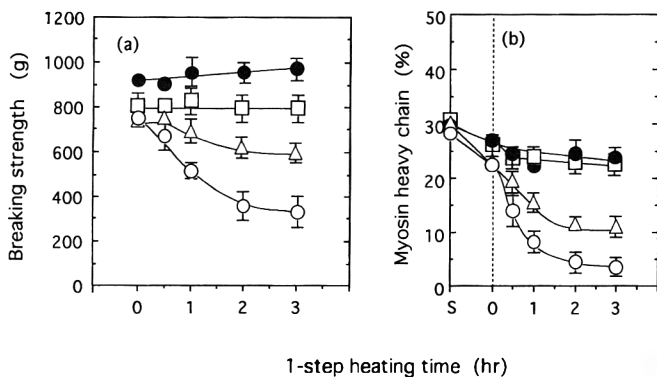
Fig. 5—Effect of bovine plasma powder on breaking strength and myosin heavy chain content of 2-step heating gel. Salted surimi pastes containing 0 (○), 5 (△), 10 (□), and 20 (●) mg/g bovine plasma powder were incubated at 30°C for 0–3 hr and heated at 90°C for 30 min. The breaking strength (a) and myosin heavy chain content (b) of 2-step heating gels were measured and plotted against the 1-step heating time. S, salt-ground meat; 0, 1-step heating gel at 90°C. Each point is the mean of six determinations. Vertical bars represent ± standard deviation.

breakdown of myosin heavy chain was effectively depressed by adding ≥10 mg of bovine plasma powder/g surimi paste. In addition (Fig. 6), no gel degradation was observed during incubation of surimi paste containing bovine plasma powder (10 mg/g) at 30–60°C, and proteolytic degradation was also depressed (data not shown). As compared with other results (Fig. 4 and 6), the effect of bovine plasma powder on gel formation of chum salmon surimi was almost the same as that of E-64. Thus, the bovine plasma powder seemed to act as a cysteine protease inhibitor.

In our study, the salted surimi paste was stuffed into a casing (22 mm diam) and placed into a hot water bath (90°C) for gelation. Accordingly, its internal temperature reached the prescribed temperature within about 15 min. However, it would have taken much longer for the product temperature to reach 80°C, if the salted surimi paste was stuffed into a larger casing and cooked in a dry oven, often used in commercial surimi-based production. In such case, endogenous proteases may cause protein degradation during cooking. Thus, a technique to inhibit protein degradation is needed to manufacture surimi-based products. Our results clearly showed that bovine plasma powder was an effective protease inhibitor to prevent proteolysis of chum salmon surimi. We concluded that the 1-step heating at higher temperatures appeared to be the best treatment for maximum gel strength of chum salmon surimi with bovine plasma powder.

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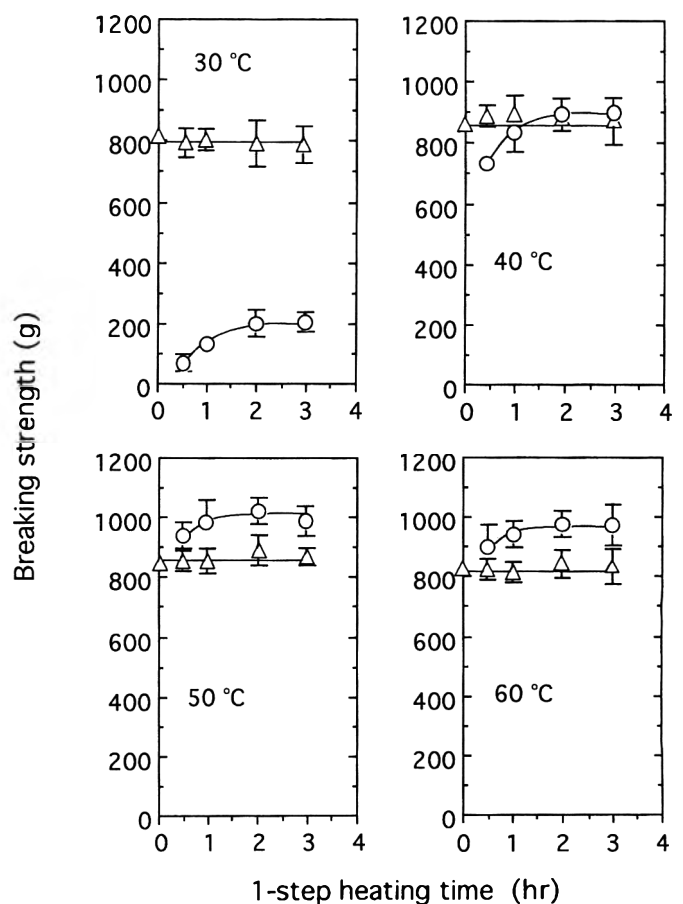
Fig. 6—Effect of bovine plasma powder on breaking strength at various 1-step heating temperatures. The breaking strength of 1-step heating gel (○) and 2-step heating gel (△) containing 10 mg of bovine plasma powder/g salted surimi paste was plotted against the 1-step heating time at 30, 40, 50, and 60°C. Each point is the mean of six determinations. Vertical bars represent ± standard deviation.

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Electrical Conductivity of Pacific Whiting Surimi Paste during Ohmic Heating

J. YONGSAWATDIGUL, J.W. PARK, and E. KOLBE

──────── ABSTRACT ────────

Electrical conductivities of Pacific whiting surimi paste with various moisture contents (75, 78, 81, and 84%) and added salt (1, 2, 3, and 4%) were measured using ohmic heating at alternating current of 3.3, 6.7, and 13.3 V/cm. Electrical conductivity of surimi increased with temperature and salt content and slightly increased with moisture content. Electrical conductivity correlated linearly with temperature ($r^2 \cong 0.99$). Generally, voltage gradient did not affect conductivity. However, variations of conductivity with voltage gradient observed in surimi containing 3–4% salt, were probably caused by electrochemical reactions at electrode surfaces. The empirical model of electrical conductivity predicted values $\pm 16\%$ of independent experimental results.

Key Words: ohmic heating, electrical conductivity, surimi, whiting

INTRODUCTION

SURIMI made from Pacific whiting (*Merluccius productus*) normally undergoes textural degradation due to the presence of a heat-stable endogenous protease in the flesh (Chang-Lee et al., 1989; Patashnik et al., 1982). The protease has been identified as cathepsin L with an optimum temperature at 55°C (Seymour et al., 1994). A breakdown of myofibrillar proteins, caused by proteolysis, inhibits proper development of a three-dimensional gel structure (Niwa, 1992). As a result, the gel forming ability of Pacific whiting surimi is low unless food grade enzyme inhibitors, such as beef plasma proteins, egg white, and potato extract are added (Chang-Lee et al., 1989; Morrissey et al., 1993; Porter, 1992).

In conventional heating methods, heat is transferred from the heating medium to the product interior by means of both convection and conduction. Yongsawatdigul et al. (1995) illustrated that temperature at the center of a cylindrical sample (I.D. = 1.9 cm, length = 17.9 cm) of whiting surimi paste heated in a 90°C water bath took ≈10 min to reach 70°C. The protease is completely inactivated at that temperature (Seymour et al., 1994). For two min of this process, surimi was exposed to a temperature range in which the protease is most active: 40–60°C. Thus, a typical slow heating rate activates the protease to degrade myofibrillar proteins before the protease can be thermally inactivated. To minimize proteolytic activity without use of enzyme inhibitors, the protease would need to be thermally inactivated quickly. This could be achieved through rapid heating methods as reported by Greene and Babbitt (1990) and Yongsawatdigul et al. (1995).

Ohmic heating is a method in which an alternating electrical current is passed through an electrically conducting product (de Alwis and Fryer, 1992). Heat is internally generated due to electrical resistance of the sample, supporting a rapid heating rate. Moreover, ohmic heating can provide uniform temperature distribution because both liquid and solid phases are heated simultaneously (Parrott, 1992). Such characteristics have led to development of commercial ohmic sterilization for particulate

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foods (Biss et al., 1989; de Alwis and Fryer, 1990). Application of ohmic heating in seafoods has also been investigated. Gel strength of surimi made from walleye pollock, white croaker, threadfin bream, and sardine was improved when samples were ohmically heated, as compared with samples heated in a 90°C water bath (Shiba, 1992; Shiba and Numakura, 1992). Pacific whiting surimi heated ohmically with an applied voltage gradient of 13.3 V/cm had good gel quality (shear stress of 30.5 kPa; shear strain of 2.8). Those gels heated conventionally had shown shear stress and strain values of 13.9 kPa and 1.3, respectively (Yongsawatdigul et al., 1995). Improved gel functionality was accompanied by retention of myosin heavy chain, indicating that the endogenous protease was quickly inactivated by rapid heating associated with ohmic heating.

To design the ohmic process optimally, electrical conductivity of food materials during ohmic heating must be elucidated. Conductivity is a critical parameter influencing the rate of heat generation (Palaniappan and Sastry, 1991a and de Alwis and Fryer, 1992). Electrical conductivities measured using alternating current (50 or 60 Hz) have been studied on various food products (Halden et al., 1990; Palaniappan and Sastry, 1991a, b; Schreier et al., 1993). However information about electrical conductivity of seafoods, particularly surimi paste, is lacking. Therefore, our objectives were: (1) to investigate effects of temperature, voltage gradient, moisture, and added salt content on electrical conductivity of Pacific whiting surimi paste and (2) to establish an empirical model of electrical conductivity as a function of composition.

MATERIALS & METHODS

Surimi paste preparation

Pacific whiting (*Merluccius productus*) surimi was taken from a process line of a local manufacturer and mixed with 4% sucrose, 4% sorbitol (ICI Specialties, New Castle, DE), and 0.3% sodium tripolyphosphate (B.K. Ladenburg Corp., Cressk II, NJ) at the OSU Seafood Laboratory. No food grade enzyme inhibitors were added. Samples were frozen and kept in a −30°C room throughout the experiment. Frozen surimi samples were thawed at room temperature (~23°C) for 2 hr and cut into small pieces (about 3 cm cubes). Sixteen batches of surimi paste representing four different moisture contents (75, 78, 81, and 84% wet basis) and added NaCl (Mallinckrodt Inc., Paris, KY) (1, 2, 3, and 4% w/w) were randomly prepared. One kg of partially thawed surimi was chopped for 1.5 min in a Stephan vertical vacuum cutter (model UM 5 universal, Stephan Machinery Co., Columbus, OH). Salt was added and mixed with surimi for another 1.5 min. Then ice was added to adjust final moisture content to the desired level and the sample was further chopped at high speed under vacuum of 600 mmHg for 3 min. Final moisture content of each batch was determined using the standard oven method (AOAC, 1984). The paste was stuffed into PVC tubes (1.9 cm i.d. × 20.5 cm long) and heated using an ohmic heating apparatus.

Electrical conductivity measurement

A sample tube containing surimi paste was placed on the sample holders (Fig. 1). An electrode was inserted into each end of the tube to provide a sample length of 15 cm. The minimum pressure that would maintain an air-free contact between the rhodium-coated stainless steel electrodes and the paste, 448 kPa, was applied to the sample. The sample was heated to 90°C using alternating current of 60 Hz at applied voltages of 50, 100, and 200 V, corresponding to voltage gradients of 3.3, 6.7,
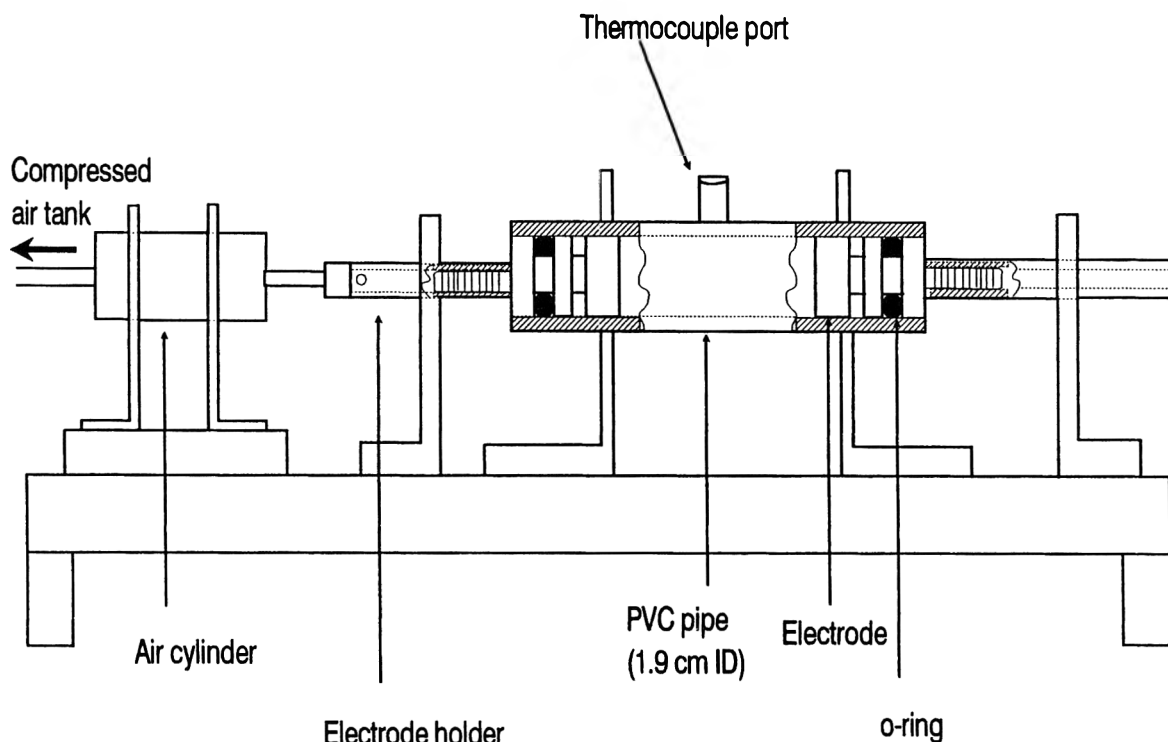
Fig. 1—Diagram of ohmic heating apparatus.

and 13.3 V/cm, respectively. Temperature at the geometric center of the samples was continuously measured with a T-type thermocouple covered with teflon to prevent interference from the electrical field. Voltage and current were measured using voltage and current transducers, respectively. Temperature, voltage and current data were recorded on a datalogger (model 21×, Campbell Scientific, Inc., Logan, UT) at 10, 2, and 1 sec intervals, according to the applied voltage gradient of 3.3, 6.7, and 13.3 V/cm, respectively. Electrical conductivity of surimi paste during ohmic heating was measured twice at each voltage gradient.

The accuracy of the voltage and current transducers was tested against a portable multimeter (John Fluke Manufacturing, Everett, WA). The voltage was applied to the circuit connected with a known 500 Ω resistor. Voltage and current transducers were calibrated to provide the same readings as those from the multimeter. Surimi paste is a homogenous material and electric field was assumed to be uniform along the sample tube, thus temperature variation within the sample tube was neglected.

Data analysis

Electrical conductivities of the samples were calculated from voltage and current data using the equation described by Palaniappan and Sastry (1991a):

$$\sigma = (1/R) (L/A)$$

where, σ = electrical conductivity (S/m); L = sample length (m); A = cross-sectional area of the sample (m²); R = resistance of the sample (ohm).

A large number of the data points (6,073) were obtained from 96 measurements and thus the data splitting technique (Bowerman and O'Connell, 1990) was used to develop and validate the empirical model. One-third of the experimental runs (32 runs, 1,849 data points) were randomly separated and used to validate the model, which was developed from the rest of the data using a Statistical Analysis System computer program (SAS Institute, Inc., 1990). Nine variables including three main effects temperature (T), moisture content (M), and added NaCl content (N), three interactive effects (TM, TN, MN), and second order polynomial terms (T², M², N²), were initially analyzed for statistical significance using stepwise and backward regression. Residual plot of the model was evaluated to assure that the assumption of constant variance and normality were satisfied.

RESULTS & DISCUSSION

SURIMI is washed and dewatered minced flesh consisting primarily of myofibrillar proteins. Due to three cycles of washing and dewatering, ionic constituents originally present in the fish are removed with wash water, resulting in a low ion content of surimi (Lin, 1992). The sodium content of Pacific whiting surimi without enzyme inhibitors was 0.002% (Chung et al., 1993); this is relatively low considering the amount of NaCl used (1–4%). For this reason, the effect of added NaCl content was studied instead of total ion content.

Linear relationships between temperature and electrical conductivity were evident (r² 0.987 to 0.999, Table 1). Electrical conductivity of Pacific whiting surimi paste changed with respect to temperature at various added NaCl (Fig. 2) and moisture contents (Fig. 3). Since variation between two replicates (Table 1) was low, the raw data from only one were presented. Electrical conductivity increased as temperature and added salt content increased, and slightly increased as moisture content increased.

As salt content increased, the ions (Na⁺ and Cl⁻) for conducting electrical current increased, which increased conductivity. Increased conductivity at high temperature was due to ionic mobility (Palaniappan and Sastry, 1991a). This linearly increasing trend agreed with results of Fryer et al. (1993) and Palaniappan and Sastry (1991a, b).

However, electrical conductivity of purified proteins, such as wool, collagen, and elastin varied with temperature according to an Arrhenius-type equation (Pethig, 1979). Differences in the trend could be due to differences in electrical conducting mode of the materials. Ionic constituents were important in conducting electrical currents through the surimi paste and other food materials during ohmic heating. On the other hand, electrical conductivity in the purified proteins (Pethig, 1979) was mainly attributed to negative and positive charges associated with the protein molecules. According to Shiba (1992), impedance of surimi paste linearly decreased as it was heated ohmically from 5°C to 50°C, indicating an increase of electrical conductivity with increased temperatures. However, electrical conductivity of surimi above 50°C is not directly comparable with our results because electrical current in Shiba's (1992) study was controlled below 3 A to achieve a desired heating rate.

Electrical conductivity tended to increase with moisture content (Fig. 3). Ion solvation could increase when more water molecules are available, resulting in increased ionic mobility. An

Table 1—Means of estimated parameters of electrical conductivity modeled as a function of temperature

Moisture (%)	Salt (%)	Coeff. (°C)$^{-1}$ Voltage gradient (V/cm)			Intercept (S/m) Voltage gradient (V/cm)		
		3.3	6.7	13.3	3.3	6.7	13.3
75	1	0.030±0*	0.030±0	0.028±0	0.540±0.003	0.606±0.039	0.649±0.062
	2	0.052±0.002	0.049±0	0.050±0.001	1.099±0.041	1.204±0.007	1.267±0.033
	3	0.074±0.001	0.067±0.004	0.069±0.002	1.704±0.018	1.904±0.020	1.921±0.037
	4	0.092±0	0.090±0	0.104±0.003	2.264±0.028	2.533±0.009	2.160±0.071
78	1	0.033±0.001	0.031±0	0.029±0.001	0.613±0.007	0.645±0.025	0.696±0.024
	2	0.053±0.001	0.051±0.001	0.051±0.004	1.257±0.014	1.326±0.026	1.449±0.042
	3	0.080±0	0.072±0.002	0.087±0.003	1.653±0.059	1.869±0.032	1.894±0.042
	4	0.099±0.003	0.096±0.001	0.100±0	2.285±0.176	2.651±0.069	2.709±0.065
81	1	0.032±0.001	0.031±0	0.029±0.001	0.629±0.009	0.682±0.034	0.767±0.010
	2	0.058±0	0.054±0	0.057±0.002	1.327±0.016	1.381±0.038	1.499±0.028
	3	0.083±0.003	0.076±0.003	0.082±0.003	1.933±0.023	2.239±0.256	1.984±0.079
	4	0.112±0.005	0.110±0.003	0.131±0.001	2.540±0.046	2.556±0.046	2.404±0.125
84	1	0.036±0.001	0.034±0.001	0.032±0	0.678±0.034	0.758±0.038	0.832±0.024
	2	0.060±0.001	0.057±0	0.056±0	1.433±0.028	1.623±0.004	1.708±0.008
	3	0.074±0.001	0.081±0.001	0.088±0.002	2.006±0.056	2.433±0.062	2.418±0.301
	4	0.092±0.002	0.107±0.001	0.106±0.008	3.343±0.107	3.495±0.112	3.051±0.268

* 0 indicates values less than 0.001.
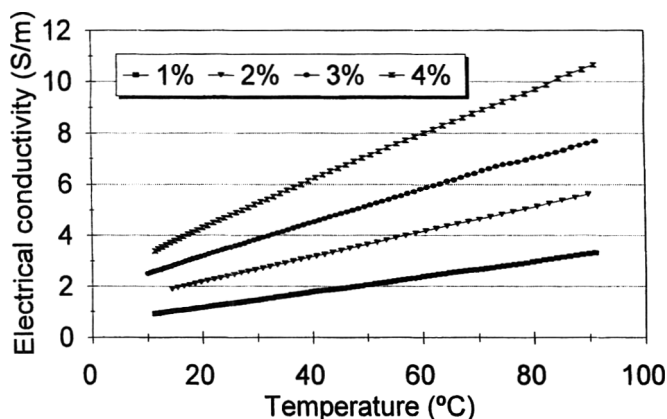
Fig. 2—Effect of salt content on electrical conductivity of surimi paste containing 75% moisture content, measured at 6.7 V/cm.
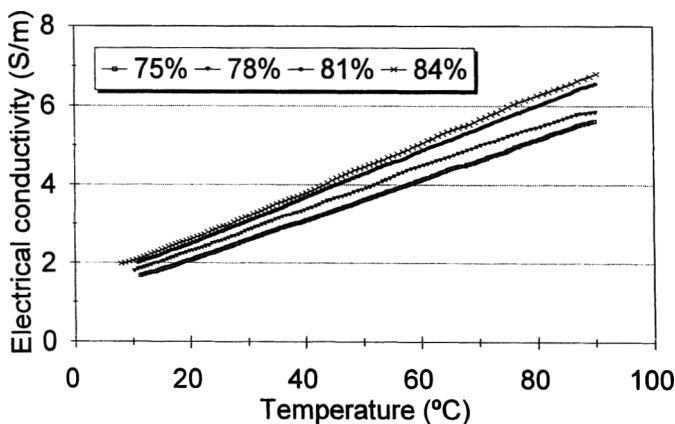
Fig. 3—Effect of moisture content on electrical conductivity of surimi containing 2% salt at voltage gradient of 3.3 V/cm.

Fig. 4—Effect of voltage gradient on electrical conductivity. (a) 75% moisture and 4% salt; (b) 84% moisture and 3% salt.

increase in electrical conductivity with increased moisture was also reported in various proteins, such as hemoglobin, keratin, and bovine plasma albumin (Pethig, 1979). This was due to ionic conduction effects associated with salts in hydrated proteins. Such hydration allowed protons and other ionic species to migrate around the surface of the proteins (Pethig, 1979). Our study demonstrated that changes in electrical conductivity were greater with respect to salt content than moisture content in the studied ranges.

Temperature coefficients at various voltage gradients of each moisture-salt combination were very similar (Table 1), indicat-

ing that the voltage gradients had no effect on electrical conductivity. However, the effect of voltage gradient was notable in samples containing 3–4% NaCl (Figs. 4a-b). The effect of voltage gradient on electrical conductivity of fruit and vegetable products was reported by Palaniappan and Sastry (1991b) and Halden et al. (1990). High applied electric field enhanced cell fluids motion within plant cells and ruptured cell membranes, resulting in release of cell fluids (Halden et al., 1990). Electrical conductivity of fruits and vegetables increased with increasing voltage gradient. Differences in electrical conductivity at varied voltage gradients we observed were unlikely to have arisen from the enhanced motion of the cell fluids. Surimi paste was finely comminuted and was a homogeneous material which was quite different from vegetables or fruits that retained the integrity of

plant cells during ohmic heating. Dependence of electrical conductivity on voltage gradient could have been caused by corrosion of the electrodes which often occurred in samples containing 3 and 4% NaCl (Figs. 4a-b). Faradaic current generated by electrolytic reactions was observed when current density >3,000 A/m² passed in a platinized-titanium electrode immersed in saturated sodium chloride solution (Stirling, 1987). Thus, an electrical current measured under corroded conditions included both applied alternating current and Faradaic current (Oldham and Myland, 1994). To minimize corrosion of the electrode, the maximum current density was recommended at 4,000 A/m² (de Alwis and Fryer, 1992).

Relationships between applied voltage and electrical current of surimi pastes at 25°C were studied (Fig. 5). For the sample containing 4% NaCl and 84% moisture, electrode corrosion and deviation from Ohm's law were observed when electrical current exceeded 1 A, corresponding to a current density of about 3,500 A/m². However, Ohm's law was followed for the sample containing 1% NaCl and 75% moisture, when the total current was <1 A and electrode corrosion was not noticed. To obtain accurate electrical conductivity values, it is critical to measure conductivity such way that electrochemical reactions at the electrodes and any other polarization phenomena do not occur.

A model was developed for electrical conductivity of Pacific whiting surimi as a function of temperature (T), added NaCl content (N) and moisture content (M). Voltage gradient was not included because it did not appear to affect electrical conductivity. The empirical model can be written as:

$$\sigma = 0.1168 + 0.0083T - 2.5115N + 0.0385MN + 0.0229TN + 0.0282N^2$$

where σ = Electrical conductivity (S/m); N = Salt content (% w/w); M = Moisture content (% wet basis); T = Temperature (°C).

All variables were significant ($P < 0.001$). Positive coefficient of TN indicated that an increase in electrical conductivity with temperature was greater at higher salt content. Although the effect of moisture content was not significant, a positive coefficient of MN suggested that an increase in electrical conductivity with salt content was greater in samples with higher moisture content. Furthermore, electrical conductivity increased quadratically with salt content. Statistical insignificance of T^2 confirmed that electrical conductivity of whiting surimi pastes, with compositional characteristics in these ranges, linearly increases with temperature. Predictability of the model was illustrated (Fig. 6a-b). The model satisfactorily predicted electrical conductivity of Pacific whiting with an error ranging from 0–16%. Error of prediction was relatively large in the sample containing 3–4% NaCl and 84% moisture (Fig. 6b). This was probably due to the electrode corrosion problem. Although the model was primarily developed for Pacific whiting surimi paste, it could be used to estimate electrical conductivity of surimi from other species. This inference was based on the fact that ionic content of surimi from various fish could be similar due to washing and dewatering. Furthermore, electrical conductivity of surimi-based seafoods, such as, imitation crabmeat, could be predicted from the model because the main factors affecting electrical conductivity of such products are only salt and moisture content.

CONCLUSION

ELECTRICAL CONDUCTIVITY was highly dependent on temperature and added salt content and slightly dependent on moisture content. The effect of applied voltage was insignificant. Changes of electrical conductivity affected by heating and compositional characteristics indicated the importance of such factors in design and operation of ohmic heaters for surimi-based seafood products. The empirical model adequately predicted the electrical conductivity of whiting surimi based on temperature, salt and moisture.
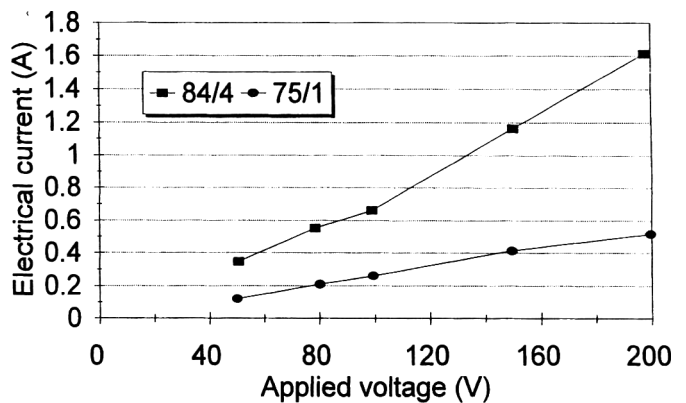
Fig. 5—Polarization of electrodes: 84/4: surimi paste containing 84% moisture and 4% salt at 25°C; 75/1: surimi paste containing 75% moisture and 1% salt at 25°C.
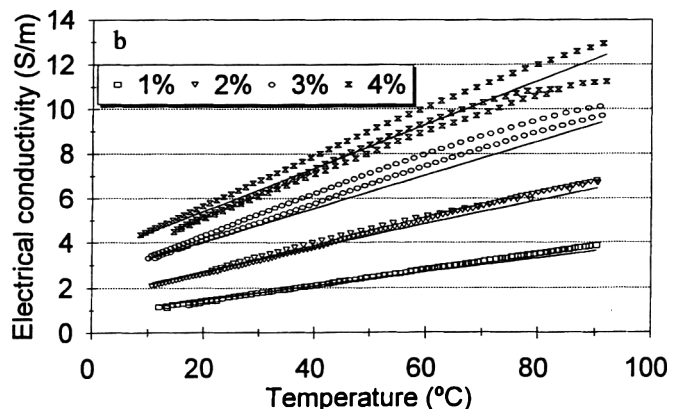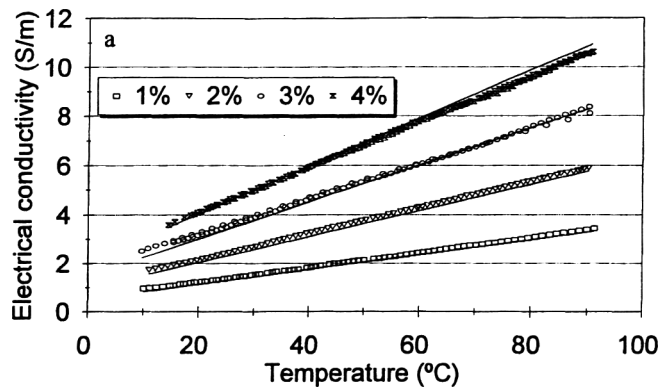
Fig. 6—Predictability of the electrical conductivity model as compared with experimental values. (a) 75% moisture; (b) 84% moisture; line: predicted values; symbol: experimental values.

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Biogenic Amines in the Flesh of Sailfish (*Istiophorus platypterus*) Responsible for Scombroid Poisoning

DENG-FWU HWANG, SHENG-HSIUNG CHANG, CHYUAN-YUAN SHIAU, and CHANG-CHIA CHENG

———————————— ABSTRACT ————————————

Sailfish fillets were involved in a 1994 food poisoning outbreak in western Taiwan. Samples were collected from the victims' residues and from retail and wholesale suppliers. They were analyzed for amines by a gradient elution system of HPLC. Tryptamine, histamine, spermine, trimethylamine, spermidine, and cadaverine were found; other amines were not detectable. The concentration of histamine was higher than 100 mg/100g which was assumed as the toxic level. The allergy-like symptoms of the victims along with the high content of histamine in the sailfish they had eaten were reasonable evidence that the food poisoning was directly associated with histamine intoxication.

Key Words: histamine, amines, food poisoning, sailfish, scombroid

INTRODUCTION

AN INCIDENT OF FOOD POISONING due to ingesting the flesh of sailfish (*Istiophorus platypterus*) occurred in Changhua Prefecture, western Taiwan, in July 1994. The incident caused illness of 12 victims from five families. Symptoms appeared soon after eating sailfish fillets and included rashes, urticaria, nausea, vomiting, diarrhea, flushing, tingling and itching of the skin. The victims recovered within 8 hr. According to the survey of the Taiwan Fisheries Bureau, the suspected fillets were from a seafood supplier of Pingtung Prefecture in southern Taiwan.

The allergy-like symptoms were similar to those caused by histamine. Poisoning by histamine has historically been referred to as scombroid poisoning because of the frequent association of the illness with the consumption of spoiled scromboid fish such as tuna and mackerel. The fishes usually implicated in episodes of histamine poisoning are Scombridae, Scomberesocidae, Pomatomidae, Coryhaenidae, Carangidae, Clupeidae and Engraulidae (Taylor, 1986). In addition to the above fish families, black marlin (*Makaira mazara*) and striped marlin (*M. audax*) belonging to Istiopheridae have also been involved in outbreaks of scombroid poisoning in Japan and USA (Taylor, 1986; Russell and Maretic, 1986). In Taiwan, scombroid poisoning has also occurred occasionally (Murray and Hobbs, 1982; Chen and Malison, 1987; ROCDH, 1988, 1992), and the fishes implicated in outbreaks were tuna, mackerel, and black marlin. However, sailfish (*I. platypterus*) has never been reported as a histamine poisoning fish.

Our objective was to investigate the association of the food poisoning incident with the amine levels of sailfish fillets obtained from victims' residues. Outbreaks of histamine poisoning after eating fish are mainly due to poor quality of raw material or poor handling. Therefore, sailfish fillets from the same species were also collected from the retail supplier in Changhua Prefecture and the wholesale supplier in Pingtung Prefecture and analyzed for amine levels.

MATERIALS & METHODS

Reagents

Standard amines, including tryptamine hydrochloride, 2-phenylethylamine hydrochloride, putrescine dihydrochloride, cadaverine dihydroch-

loride, spermidine trihydrochloride, spermine tetrahydrochloride, histamine dihydrochloride, trimethylamine hydrochloride, tyramine hydrochloride and agmatine sulfate, were obtained from Sigma Chemical Company (St. Louis, MO). Methanol (LC grade) and other reagents (GR grade) were from E. Merck (Darmstadt, Germany).

Samples

A total of five samples were collected. Two samples, including frozen (288g) and fried (176g) fillets, were the residues of one of the victims. The other three samples (492, 296, and 2100g) were frozen fish fillets from the same specimen of sailfish (*Istiophorus platypterus*) obtained from fish shops in Changhua and Pingtung Prefecture, which were the retail and wholesale suppliers of the fillets causing the food poisoning. The sailfish had been caught in the adjacent coastal water of Pingtung Prefecture and its weight was about 50 kg. The wholesale supplier bought it and cut it into fillets. The fillets were then distributed to retail suppliers. Of the five samples, one from the retail supplier had a yellow-green surface color; the others were pink. All samples were wrapped and kept at −20°C until assayed.

Preparation of standard amine solution

Tryptamine hydrochloride (122.8 mg), 2-phenylethylamine hydrochloride (130.1 mg), putrescine dihydrochloride (182.9 mg), cadaverine dih-
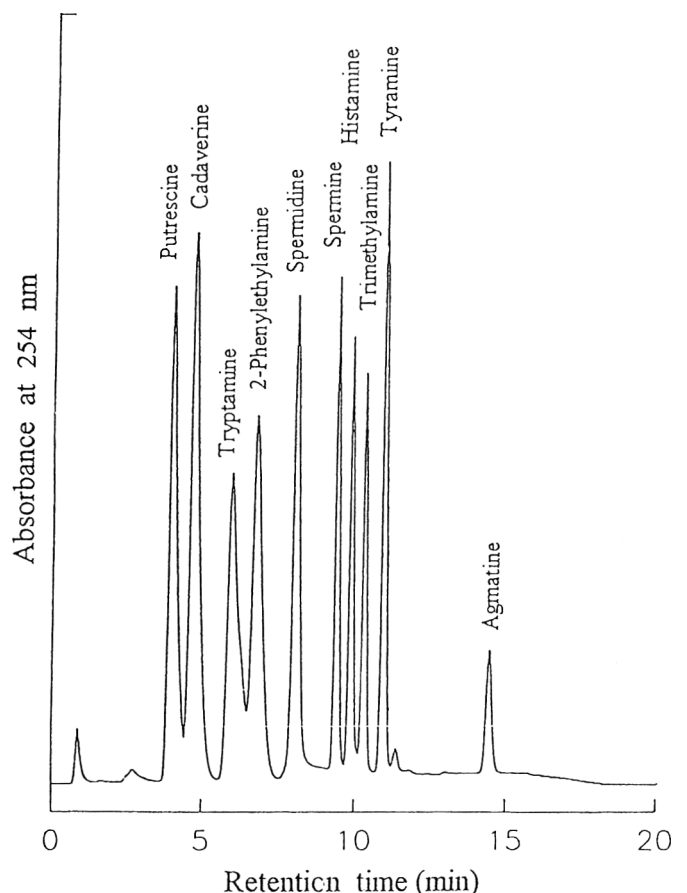
Fig. 1—HPLC profile of authentic amines.

ydrochloride (171.4 mg), spermidine trihydrochloride (175.3 mg), spermine tetrahydrochloride (172.0 mg), histamine dihydrochloride (165.7 mg), trimethylamine hydrochloride (161.7 mg), tyramine hydrochloride (126.7 mg) and agmatine sulfate (175.4 mg) were dissolved in 10 mL deionized water and used as standard samples. The final concentration of each amine (free base) was 10 mg/mL solution.

Benzoylation of standard amine solution

The benzoyl derivatives of amines were prepared according to the method of Yen and Hsieh (1991) with minor modifications. To the standard amine solution (50 µL), 2M sodium hydroxide (1 mL) was added, followed by 10 µL of benzoyl chloride. This was mixed by using a vortex mixer and allowed to stand for 20 min. The benzoylation was stopped by adding 2 mL saturated NaCl solution and the amide was extracted with 3 mL diethyl ether. After centrifugation, the upper organic layer was transferred into a tube and evaporated to dryness in a stream of nitrogen. The residue was dissolved in 500 µL methanol and 5-µL aliquots were injected for HPLC analysis.

Sample preparation and amine extraction

After partially thawing, each fillet was ground in a Waring Blendor for 3 min. Ground sample (5g) was transferred to a 50-mL centrifuge tube and homogenized with 20 mL of 6% trichloroacetic acid for 3 min. The homogenate was centrifuged (12,000 rpm, 10 min, 4°C) and filtered through Whatman No. 2 filter paper. The filtrate was placed in a volumetric flask and made up to 50 mL. Each extract (2 mL) was derivatized with benzoyl chloride using the same procedure as for the benzoylation of standard amine solutions.

Apparatus

Amines were determined by using a Hitachi Liquid Chromatograph (Hitachi, Ltd., Tokyo) consisting of a Model L-6200 pump, a Rheodyne Model 7125 syringe loading sample injector, a Model L-4000 UV-VIS detector set at 254 nm, and a Model D-2500 Chromato-integrator. A Lichrospher 100 RP-18 reverse-phase column (5 µm, 125 × 2.5 mm i.d., E. Merck) was used for separation.

Chromatographic conditions

The gradient elution program was set at 1.1 mL/min, starting with a methanol-water mixture (55:45, v/v) for 4.0 min. The program proceeded linearly to methanol-water (80:20, v/v), with a flow rate of 1.1 mL/min over 2.0 min. This was followed by the same composition and flow rate for 6.0 min, then decreased over 2 min to methanol-water (55:45, v/v) at 1.1 mL/min.

Determination of trimethylamine oxide

The peak of trimethylamine (TMA) in the HPLC chromatogram might include free TMA derived from the reduction of trimethylamine oxide (TMAO) in the benzoylation procedure. Thus, TMAO was further measured by using the method of Wekell and Barnett (1991). Briefly, to each amine extract (10 mL) was added toluene and Fe-EDTA reagent, followed by incubation in a water bath for 5 min at 50°C. After cooling, the sample was mixed with 45% KOH, dehydrated with anhydrous sodium sulfate, reactivated with picric acid solution, and absorbance was measured at 410 nm with a UV 2000 Spectrophotometer (Hitachi).

RESULTS & DISCUSSION

A TYPICAL CHROMATOGRAPHIC PROFILE of 10 standard biogenic amines by the gradient elution system was developed (Fig. 1). All 10 amines were well separated in 15 min total run time with good peak resolution, sharpness and symmetry. Standard curves of 10 amines were separately prepared in the range of 0–0.1 mg/100 mL and peak area vs amount of amine was plotted. Data for standard curves were subjected to linear regression analysis. The correlation coefficients and linear regression coefficients for each amine were compared (Table 1). The correlation coefficient in every curve was 0.99. This indicated a definite linear relationship between amine concentration and detector response. We concluded that the gradient elution program was satisfactory. Recoveries of amines by the sample extraction procedure were

Table 1—Linear regression equation* and correlation coefficients for authentic amines

Amines	Linear equation coefficient a	Linear equation coefficient b	Correlation coefficient
Putrescine	−11031	251415	0.99974
Cadaverine	−26336	333694	0.99836
Tryptamine	−20187	232916	0.99751
2-Phenylethylamine	−20054	232893	0.99750
Spermidine	−6456	198311	0.99771
Spermine	15272	129211	0.99229
Histamine	808	118699	0.99857
Trimethylamine	−9838	126459	0.99451
Tyramine	−11122	181602	0.99556
Agmatine	−23066	118238	0.99468

* $Y = a + bX$, where Y = relative peak area and X = amount of amine injected into HPLC (µg).
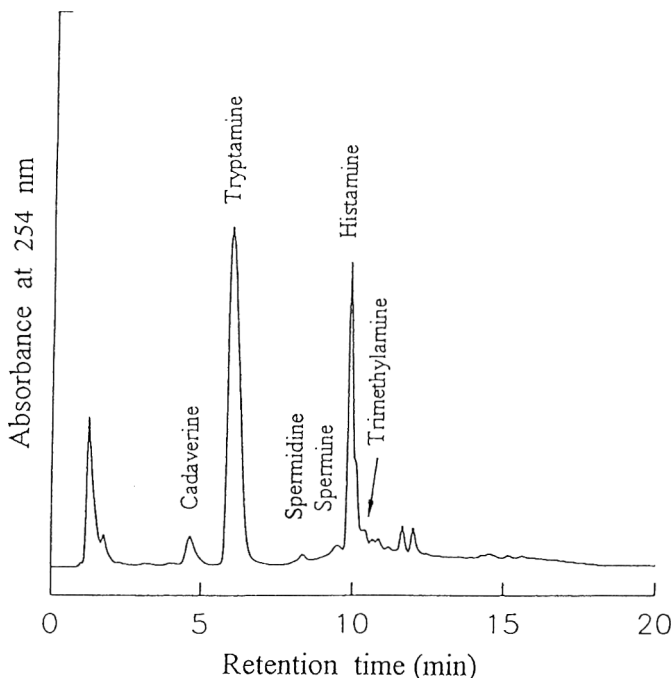
Fig. 2—HPLC profile of amines in fried fish fillet from victims' source.

101% for putrescine, 99.4% for cadaverine, 86.4% for tryptamine, 101% for 2-phenylethylamine, 92.6% for spermidine, 90.2% for spermine, 86.4% for histamine, 88.3% for tyramine, 94.3% for TMA and 98.5% for agmatine.

The typical HPLC profile of amines from a sailfish fillet was developed (Fig. 2), and the amine levels of all samples were summarized (Table 2). Tryptamine, histamine, spermine, TMA, spermidine and cadavarine were predominant in all samples; other amines were not detectable (<2.5 mg/100 g). Tryptamine, histamine, spermine, TMA, spermidine and cadavarine in all fillet samples ranged from 75.0–207.5, 32.5–180.0, <2.5–50.0, 12.5–28.5, <2.5–17.5 and 6.0–14.5 mg/100g, respectively. All samples, except the fillet with yellow-green color, contained histamine >100 mg/100g. TMAO levels measured by the colorimetric method were <4.0 mg/100g in all samples. Therefore, we concluded that the peak area of TMA in the HPLC chromatogram for each sample represented the amount of TMA.

Although scombroid poisoning may be caused by many factors other than histamine itself (Geiger, 1955; Arnold and Brown, 1978; Clifford et al., 1989), histamine concentration is considered a good indicator of fish deterioration and scombroid poisoning (Russell and Maretic, 1986; Taylor, 1989). The U.S. Food & Drug Administration (FDA) has established a hazard action level for histamine in fish of 50 mg of histamine/100g fish (500 ppm) based on data collected from numerous outbreaks

Table 2—Concentration of amines in the flesh of sailfish (*Istiophorus platypterus*) causing food poisoning

Source	Sample	Weight (g)	Amine level (mg/100g)						Outward appearance
			Cadaverine	Tryptamine	Spermine	Spermidine	Histamine	Trimethylamine	
Victim	Frozen fish fillet	288	14.5 ± 0.3*[1]	208 ± 6	50.0 ± 3.9	17.5 ± 0.6	168 ± 8	24.5 ± 3.3	Normal
	Fried fish fillet	176	11.0 ± 0.4	185 ± 11	20.0 ± 1.6	12.5 ± 1.0	180 ± 9	19.0 ± 0.5	Normal
Fish shop in Changhua	Frozen fish fillet No. 1	492	7.5 ± 0.2	213 ± 8	20.0 ± 5.3	10.0 ± 0.5	165 ± 2	18.5 ± 1.3	Normal
County	No. 2	296	6.0 ± 0.2	75.0 ± 5.5	<2.5	<2.5	32.5 ± 3.5	12.5 ± 0.2	Yellow-green
Fish shop in Pingtung	Frozen fish fillet Center part	2100	12.0 ± 0.2	190 ± 12	45.0 ± 9.4	15.0 ± 1.9	178 ± 6	28.5 ± 0.7	Normal
County	Side part		12.5 ± 0.4	160 ± 9	10.0 ± 0.4	17.5 ± 0.6	165 ± 10	25.0 ± 0.8	Normal

*[1] Mean ± S.D. for triplicate assays.

(Taylor, 1989). A survey was taken on scombrotoxic fish poisoning in Britain of over 250 suspected incidents from 1976 to 1986. Bartholomev et al. (1987) demonstrated that histamine >100 mg/100g would be toxic and unsafe for consumption. We concluded from these guidelines that the histamine content in the flesh of sailfish was >100 mg/100g and probably caused the poisoning. The allergy-like symptoms of the victims along with the high concentrations of histamine in the fillet residues indicated that the food poisoning incident occurred in Changhua, Taiwan, was caused by histamine.

A study by Clifford et al. (1989) showed that histamine administered alone was less toxic than an equal amount of histamine consumed with fish. This discrepancy might indicate the presence of other synergistic biogenic amines, like cadaverine and putrescine (Bjeldanes et al., 1978). In addition to histamine, tryptamine, spermine, TMA, spermidine and cadaverine were found in the sailfish fillets. The possible contribution of these amines on the potentiation of histamine toxicity should be considered for future studies.

The production of histamine in fish is usually associated with spoilage (Eitenmiller et al., 1982). According to reports by the victims, the sailfish fillets that caused the intoxication did not appear unpleasant or have a spoiled flavor at the time of purchase. Histamine formation in fish is well known to be associated with the growth of bacteria possessing the enzyme histidine decarboxylase. In fish, several histamine-producing bacteria have been implicated as primary contributors to histamine formation. These bacteria are *Proteus (Morganella) morganii*, *P. vulgaris*, *Klebsiella pneumoniae*, *Escherichia*, *Clostridium*, *Salmonella* and *Shigella* (Taylor et al., 1978, 1979; Eitenmiller et al., 1982; Russell and Maretic, 1986; Taylor, 1986). These bacteria are capable of producing hazardous amounts of histamine in a very short period of time when fish are held at elevated temperatures. As a result, contaminated fish may not appear spoiled but still be hazardous to consume. In order to understand the relationship between histamine formation and spoilage, the causative bacteria in the flesh of sailfish will be investigated. In addition, the relationship between bacteria and sailfish fillet pink color and yellow-green color will be studied.

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Enzymatic Hydrolysis of Crayfish Processing By-products

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ABSTRACT

Ten commercial proteases (neutral and alkaline) were evaluated for hydrolysis of crayfish processing by-products (CPBs). Hydrolysis conditions were optimized for the alkaline protease Optimase™ APL-440 by response surface methodology (RSM). Two model equations were proposed with regard to effects of pH, temperature (T), time (t), enzyme/substrate (E/S) ratio, and substrate concentration (S) on the amount of 0.3M TCA soluble peptides (TSP) and degree of hydrolysis (DH). Interaction effects between pH and T were observed ($p<0.001$). Based on TSP, optimum hydrolysis conditions were determined to be pH 8–9, 65°C, 2.5 hr reaction time, 75%(w/v) substrate concentration, and 0.3% (v/w) enzyme.

Key Words: crayfish by-products, enzymes, hydrolysis, proteases

INTRODUCTION

THE LOUISIANA CRAYFISH INDUSTRY is the largest commercial crustacean aquaculture effort in the United States with harvest exceeding 45,400 tons annually. Processing plants produce over 38,600 tons of crayfish processing by-products (CPBs) annually, with recovery of only 15% (by weight) of edible tail meat (Meyers et al., 1990).

Considerable progress has been made toward better utilization of CPBs. Pigment recovered from CPBs has been utilized in mariculture of red sea bream in Japan (Meyers, 1987). Chitosan has been prepared from chitin isolated from CPBs (No et al., 1989) and utilized as a coagulant for recovery of organic compounds from seafood processing streams (No and Meyers, 1989a,b). Many recoverable volatile flavor compounds and precursors are present in CPBs (No and Meyers, 1989b; Tanchotikul and Hsieh, 1989; Cha et al., 1992). A potential exists for utilization of CPBs for production of marketable flavor extracts. Utilization of by-products for production of foods and flavorants has been proposed for other seafood industries (Joh and Hood, 1979; Burnette et al., 1983; Reddy et al., 1989; Shiau and Chai, 1990; Cha et al., 1993; Kim et al., 1994).

Proteases can be used for recovery of flavor (Haard, 1992). Protein hydrolysates from various kinds of by-products have been produced using specific proteases (O'Meara and Munro, 1984a,b; Surówka and Fik, 1992). An enzymatic process has been developed to produce flavorants from seafood processing by-products (In, 1990). Protein hydrolysates such as hydrolyzed vegetable protein and autolyzed yeast extract have been widely used as flavor enhancers (Nagodawithana, 1992). Heat treatment can be applied to protein hydrolysate to produce Maillard reaction flavor (process flavor), a technology used for production of meat and savory flavors (Dziezak, 1986).

The objectives of our study were to evaluate different commercial protease(s) for hydrolysis of CPBs into a product suitable for further processing into seafood flavor extracts and to optimize processing conditions using response surface methodology (RSM).

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MATERIALS & METHODS

Materials

Picking table crayfish processing by-products (CPBs) were obtained from a crayfish processor in St. Martinville, LA. Clean sanitized containers were provided to pickers for collection of CPBs, which were packed on ice and immediately transported to the LSU Muscle Foods Laboratory. Upon arrival, CPBs were coarsely ground using a Paoli meat/bone separator (model 23-668, Stephen Paoli International, Rockford, IL). Ground CPBs (combination of shell and mince) were either used immediately or vacuum-packaged in polyethylene bags ($\approx$ 500g per bag, Koch Supplies, Inc., Kansas City, MO) and stored at $-20°C$.

Enzymatic hydrolysis of CPBs

Frozen ground CPBs were thawed at 4°C overnight. CPBs (100g) plus 0.05M boric acid-NaOH buffer (200 mL, pH 9.0) were ground in a Waring Blendor. One hundred mL ground CPBs were placed in a 100-mL jacketed reaction vessel (Cat. No. 991760, Wheaton, Millville, NJ) and preincubated for 30 min at the desired temperature. The pH was adjusted with aqueous 6.25N NaOH or 6N HCl with final volume adjusted to 110 mL with distilled water. A specific protease was added to the vessel, and the mixture vigorously stirred during reaction using a magnetic stirrer. Aliquots (1 mL) of hydrolysate were removed at time intervals of 10, 20, 30, 60, 90, and 120 min and immediately transferred into test tubes containing 2 mL of 0.3M trichloroacetic acid (TCA) solution. These solutions were allowed to stand at ambient temperature ($\approx$23°C) for 20 min and filtered through Whatman No. 40 filter paper. A 25 µL aliquot of filtrate was mixed with 0.225 mL distilled water, 1.25 mL 0.5N NaOH solution, and 0.25 mL 1.0N Folin & Ciocalteu's phenol reagent (Sigma Chemical Co., St. Louis, MO). The resulting solution was mixed immediately, incubated at 30°C for 15 min, and then centrifuged at 2,000 × g for 10 min to remove turbid material. Supernatant absorbance was measured at 578 nm.

Estimation of degree of hydrolysis

The degree of hydrolysis (DH) was determined using a modification of the method described by Boudrant and Cheftel (1976) and was defined as follows:

$$DH = [(D'_{\text{at time } t} - D_o)/(D_{max} - D_o)] \times 100$$

where the blank, D_o (amount of 0.3M TCA soluble peptides of unhydrolyzed CPBs expressed as tyrosine) was prepared by adding 0.3M TCA solution to unhydrolyzed CPBs. D_{max} corresponded to the maximum amount of 0.3M TCA soluble peptides as tyrosine and was determined after hydrolysis of 0.1g CPBs with 4 mL 6N HCl at 110°C for 24 hr. Corrected amount of 0.3M TCA soluble peptides, $D'_{\text{at time } t}$, was determined by subtracting the amount of 0.3M TCA soluble peptides of protease from that of hydrolyzed CPBs at time t.

Table 1—Independent variables and experimental design levels expressed in coded and natural units for Optimase APL-440

	Independent variables				
Code units	pH	Temp (°C)	Time (hr)	S[a] (%)	E/S[b] (%)
−2	7	60	0.5	15	0.1
−1	8	65	1.5	30	0.2
0	9	70	2.5	45	0.3
+1	10	75	3.5	60	0.4
+2	11	80	4.5	75	0.5

[a] Concentration (w/v) of CPBs.
[b] Ratio (v/w) of Optimase APL-440 to CPBs.
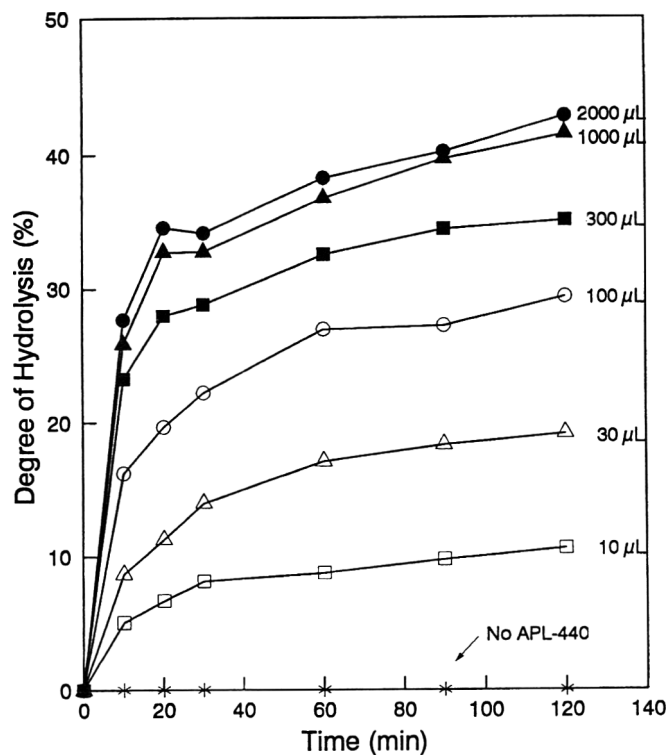
Fig. 1—Reaction progress curves for enzymatic hydrolysis of CPBs. Reaction conditions: Optimase APL-440; pH 9.0; 37°C; 30.3% (w/v) of CPBs (on a wet basis).
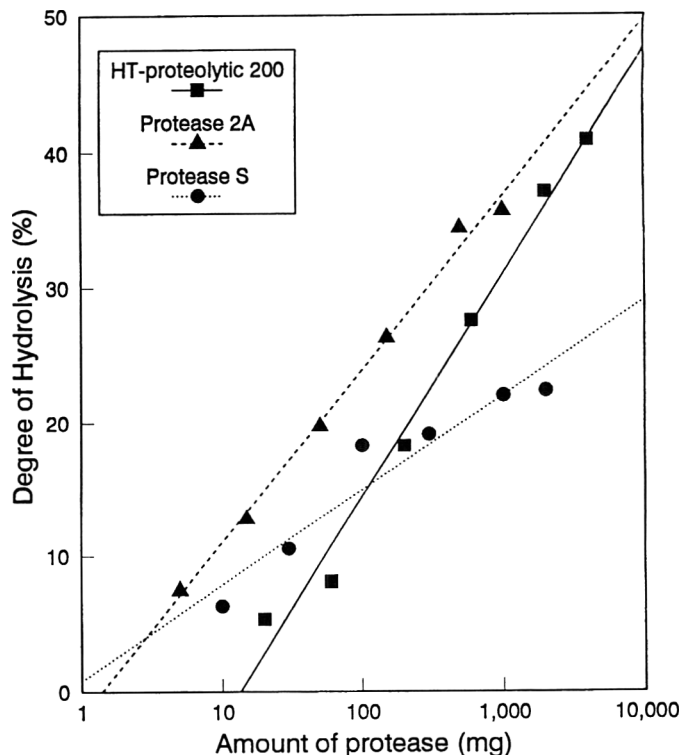
Fig. 3—Degree of hydrolysis vs $\log_{10}$ (protease amount) for neutral proteases. Reaction conditions: pH 9.0; 37°C; 30.3% (w/v) of CPBs (on a wet basis); 1 hr reaction time.
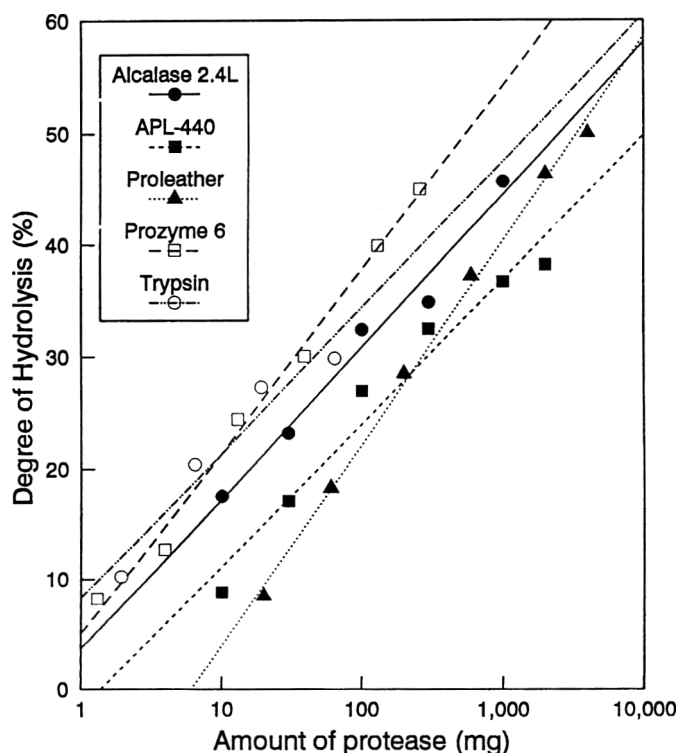
Fig. 2—Degree of hydrolysis vs $\log_{10}$ (protease amount) for alkaline proteases. Reaction conditions: pH 9.0; 37°C; 30.3% (w/v) of CPBs (on a wet basis); 1 hr reaction time.
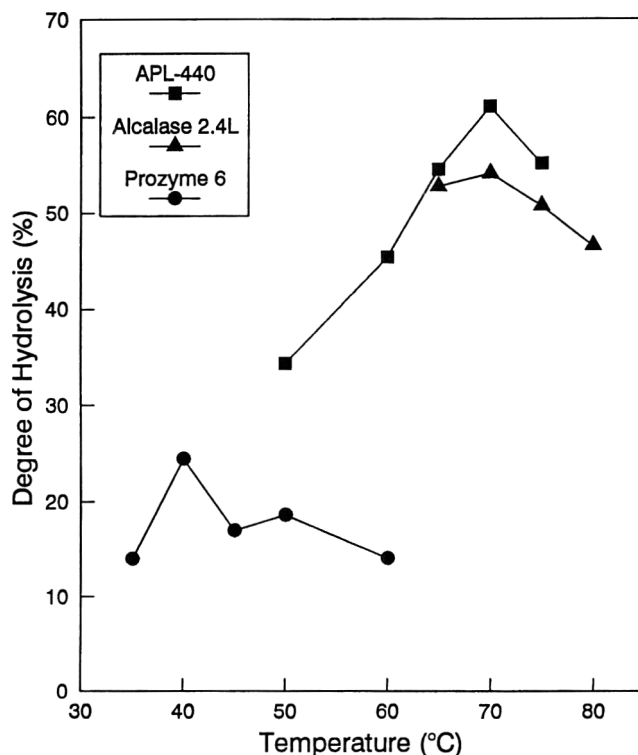
Fig. 4—Optimum temperatures for Alcalase 2.4L, Optimase APL-440, and Prozyme 6 using CPBs as substrate. Reaction conditions: pH 9.0; 100 µL of Optimase APL-440; 120 µL of Alcalase 2.4L; 10 mg of Prozyme 6; 50% (w/v) of CPBs (on a wet basis); 1 hr for Optimase APL-440 and Prozyme 6; 1.5 hr for Alcalase 2.4L.

Protease selection

Ten commercial proteases were evaluated for ability to hydrolyze CPBs. All were commercially available in bulk quantities and were supplied as free samples by manufacturers. The intrinsic pH of CPBs is generally between 8.0 and 9.0. Since it is more desirable to conduct

CPBs hydrolysis without pH adjustment, only neutral and alkaline proteases were evaluated.

Protease selection was based on the method of Hale (1969) with some modification. The plot of DH vs $\log_{10}$ (protease amount), which was

Table 2—Evaluation of commercial proteases for the hydrolysis of CPBs

Protease	Amount[a] (mg)	Activity[b]
Alkaline		
Alcalase™ 2.4L[c]	(89)	112.4
Prozyme™ 6[d]	33	303.0
Optimase™ APL-440[e]	(292)	34.2
Proleather™, [d]	270	37.0
Trypsin[f]	46	217.4
Neutral		
HT-proteolytic™ 200[e]	874	11.4
Protease 2A™, [d]	299	33.4
Papain[e]	2038	4.9
Bromelain[e]	10481	1.0
Protease S™, [d]	13512	0.7

[a] The amount of protease required for 30% hydrolysis at 37°C and 1 hr. Numbers in parentheses represent protease amount in μL.
[b] Inverse of protease amount × 10,000.
[c] Novo Nordisk Bioindustrials, Inc., Danbury, CT.
[d] Amano International Enzyme Co., Inc., Troy, VA.
[e] Solvay Enzymes, Inc., Elkhart, IN.
[f] Sigma Chemical Co., St. Louis, MO.

Table 3—Evaluation of 3 selected commercial proteases for the hydrolysis of CPBs at their optimum temperatures

Proteases	Opt. temp. (°C)	Amount[a] (mg)	Activity[b]
Optimase APL-440	70	(140)	71.4
Alcalase 2.4L	70	(143)	69.9
Prozyme 6	40	555	18.0

[a] The amount of protease required for 50% hydrolysis at optimum temperature. Numbers in parentheses represent protease amount in μL.
[b] Inverse of protease amount × 10,000.

obtained from six levels of protease, had a linear relationship. From this, the amount of protease required to degrade CPBs to a required DH in 1 hr (30% at 37°C or 50% at optimum temperature) was determined. Protease activity was expressed as the inverse of this amount.

Optimization of hydrolysis conditions

Response surface methodology (RSM) (Box et al., 1978) was used to optimize hydrolysis parameters such as initial pH, temperature (T), reaction time (t), substrate concentration (S), and enzyme/substrate ratio (E/S). Ranges of pH, T, t, and E/S were selected based on information from manufacturers and preliminary experiments. The upper substrate concentration limit was based on the highest concentration that could be effectively stirred. A range of independent variables was tested (Table 1). The coded values were obtained by the following formula:

$$Z = (X - X°)/\Delta X$$

where Z is the coded value, X is the corresponding natural value, X° is the natural value in the center of the domain, and ΔX is the increment of X corresponding to one unit of Z.

For each factor, five levels were given and a second order model was proposed. Data were analyzed by multiple regression to fit the following second order equation:

$$Y = b_0 + \sum_{i=1}^{5} b_i X_i + \sum_{i=1}^{5} b_{ii} X_i^2 + \sum_{i=1}^{4} \sum_{j=2}^{5} b_{ij} X_i X_j \quad (i<j)$$

where b_0, b_i, b_{ii}, and b_{ij} represent regression coefficients of the model, and X_i and X_j represent the independent variables in coded values. A central composite design was used requiring 36 experiments for the study of five experimental factors in coded units.

Amounts of 0.3M TCA soluble peptides (TSP) and DH were measured as dependent variables. Although amount of TSP was closely related to DH, it was chosen as one of the dependent variables since it represented real hydrolysate (short chain peptides or amino acids) produced by enzymatic hydrolysis. High DH does not necessarily result in a high amount of hydrolysate, since elevated amounts of hydrolysate can be produced with high substrate concentration even at low DH.

Statistical analysis

Response surface equations were obtained using the RSREG procedure of the Statistical Analysis System (SAS Institute, Inc., 1985) pro-

Table 4—Responses of dependent variables to the hydrolysis conditions for Optimase APL-440

Run no.	Independent variables					Dependent variables	
	pH	T	t	S	E/S	Amount of 0.3M TCA soluble peptides[a] (mg)	Degree of hydrolysis (%)
1	−1	−1	−1	−1	−1	1.40	61.0
2	1	−1	−1	−1	1	1.48	65.1
3	−1	1	−1	−1	1	1.12	45.9
4	1	1	−1	−1	−1	0.39	8.7
5	−1	−1	1	−1	1	1.48	64.9
6	1	−1	1	−1	−1	1.29	55.6
7	−1	1	1	−1	−1	1.12	45.9
8	1	1	1	−1	1	0.43	9.9
9	−1	−1	−1	1	1	2.37	51.1
10	1	−1	−1	1	−1	2.35	50.5
11	−1	1	−1	1	−1	1.86	37.7
12	1	1	−1	1	1	1.22	19.8
13	−1	−1	1	1	−1	2.58	55.8
14	1	−1	1	1	1	2.87	63.3
15	−1	1	1	1	1	2.50	53.4
16	1	1	1	1	−1	0.72	6.4
17	−2	0	0	0	0	1.91	54.4
18	2	0	0	0	0	0.62	8.9
19	0	−2	0	0	0	1.96	56.2
20	0	2	0	0	0	1.13	26.5
21	0	0	−2	0	0	1.98	57.4
22	0	0	2	0	0	2.09	61.2
23	0	0	0	−2	0	0.69	59.5
24	0	0	0	2	0	3.27	56.6
25	0	0	0	0	−2	1.96	56.3
26	0	0	0	0	2	2.12	61.9
27	0	0	0	0	0	2.16	63.0
28	0	0	0	0	0	2.13	61.9
29	0	0	0	0	0	2.10	60.7
30	0	0	0	0	0	2.11	61.6
31	0	0	0	0	0	2.15	62.8
32	0	0	0	0	0	2.09	60.5
33	0	0	0	0	0	2.06	60.0
34	0	0	0	0	0	2.09	60.9
35	0	0	0	0	0	2.26	66.5
36	0	0	0	0	0	2.13	62.3

[a] mL filtrate.

gram. Contour plots were drawn using PS-Plot (Polysoft, Salt Lake City, UT) to show the effect of two independent variables with all other factors at zero level.

RESULTS & DISCUSSION

Progress curves for enzymatic hydrolysis of CPBs

Reaction progress curves for enzymatic hydrolysis of crayfish processing by-product (CPBs) with and without Optimase™ APL-440 were compared (Fig. 1). This type curve was typical, exhibiting a fast reaction rate followed by a slowing. Similar progress curve shapes have been reported for enzymatic hydrolysis of fish protein (Archer et al., 1973), soy protein (Constantinides and Adu-Amankwa, 1980; Adler-Nissen, 1979), lean beef tissue (O'Meara and Munro, 1984a,b), and casein (Mannheim and Cheryan, 1990). The other nine proteases tested showed the same trend.

There are many possible explanations for the progress curve obtained. Adler-Nissen (1986) concluded that the shape of a progress curve was a result of substrate competition (a kind of product inhibition) between the original substrate and peptides produced during hydrolysis. O'Meara and Munro (1984b) explained that this type of curve shape was the result of rapid cleavage of susceptible peptide bonds during the initial stage, and slow cleavage of the less susceptible bonds at later stages. In enzymatic hydrolysis of an insoluble substrate, Archer et al. (1973) suggested that enzymes adsorbed onto an insoluble protein particle in a fast reaction, degraded polypeptide chains that were loosely bound to the surface, and acted on the more compact core protein more slowly. In enzymatic hydrolysis of CPBs, downward curvature of the progress curves may be explained by the combination of these factors since CPBs contain both soluble and insoluble proteins.

Table 5—Model coefficients[a] estimated by multiple linear regression for Optimase APL-440

Factor	Coefficients	
	Amount of 0.3M TCA soluble peptides[b] (mg)	Degree of hydrolysis (%)
Constant	2.14	62.40
Linear		
pH	-0.26***	-9.48***
T	-0.34***	-12.46***
t	0.04	0.96
S	0.54***	-1.03
E/S	0.09**	2.62**
Quadratic		
pH^2	-0.23***	-8.16***
T^2	-0.16***	-5.74***
t^2	-0.04	-1.25
S^2	-0.06	-1.56
(E/S)^2	-0.04	-1.30
Interactions		
pH × T	-0.25***	-8.74***
pH × t	-0.07	-2.08
pH × S	-0.04	-1.28
pH × E/S	0.05	1.38
T × t	-0.03	-0.52
T × S	-0.08*	2.05
T × E/S	0.04	0.55
t × S	0.06	1.51
t × E/S	0.09*	0.24
S × E/S	0.07	1.41
R^2	0.9696	0.9606
F	23.94	18.27
Probability > F	0.0000	0.0000

[a] Model on which X_1 = pH, X_2 = Temperature (T), X_3 = Reaction time (t), X_4 = Substrate concentration (S), X_5 = The ratio of Optimase APL-440 to substrate (E/S) is

$$Y = b_0 + \sum_{i=1}^{5} b_i X_i + \sum_{i=1}^{5} b_{ii} X_i^2 + \sum_{i=1}^{4} \sum_{j=2}^{5} b_{ij} X_i X_j \ (i<j)$$

[b] mL filtrate.
*** Significant at 0.001 level.
** Significant at 0.05 level.
* Significant at 0.10 level.

No hydrolysis occurred when CPBs were incubated without Optimase APL-440, indicating lack of remaining proteolytic activity in CPBs. Kim et al. (1992) purified trypsin-like enzymes from the hepatopancreas of crayfish. However, it has been reported that blanching crayfish for 7 min in boiling water was sufficient for inactivation of the hepatopancreatic proteases (Marshall et al., 1987). In a commercial crayfish processing plant, heat treatment is employed to facilitate peeling of crayfish for tailmeat recovery and to prevent enzymatic softening of tailmeat texture.

Selection of protease for enzymatic hydrolysis of CPBs

Researchers have used many different enzyme selection procedures for enzymatic hydrolysis of protein (Hale, 1969; Cheftel et al., 1971; Arzú et al., 1972; Rebeca et al., 1991). O'Meara and Munro (1984a) stated a rational procedure for selection of a suitable protease. However, there is no standard method for selection of an enzyme for protein hydrolysis. Hale (1969) selected a protease based on the concentration required to reach a required DH instead of on the highest degree of hydrolysis. In our study, enzyme selection was based on the method of Hale (1969) with some modification.

To select suitable protease(s) for enzymatic hydrolysis of CPBs, enzymes should be evaluated using CPBs as substrate. Although neutral proteases showed higher activities at neutral pH, all proteases were tested at pH 9.0 because the intrinsic pH of CPBs was between 8.0 and 9.0. The addition of additives, such as acids or bases, would be undesirable in a commercial operation. For preliminary selection of enzymes, a mild temperature (37°C) was chosen at which all proteases would have sufficient activities without heat inactivation.

Plots of DH vs $\log_{10}$ (protease amount) for all proteases, except for papain and bromelain, showed linear relationships (Fig.

2 and 3). Hale (1969) measured the relative activities of 20 commercial proteases for hydrolysis of fish protein and reported that a plot of solubilization vs enzyme concentration on a logarithmic scale was linear. Papain and bromelain, instead, showed linear relationships for log-log plots of DH vs protease amount (data not shown). This difference may be due to substrate specificity.

The amount of protease required to digest 30% of CPBs in 1 hr was determined from these plots. Table 2 shows the results of primary selection at 37°C. All alkaline proteases showed higher activities than did neutral proteases. Alcalase™ 2.4L, Prozyme™ 6, and trypsin showed the highest activities of the five alkaline proteases tested. Trypsin was excluded from further consideration because of its high relative cost. Alcalase 2.4L, Prozyme 6, and Optimase APL-440 showed highest activities relative to cost. Therefore, these three proteases were considered for further evaluation at their optimum temperatures.

Optimum temperatures for Prozyme 6, Alcalase 2.4L, and Optimase APL-440 using CPBs as substrate were 40°C, 70°C, and 70°C, respectively (Fig. 4). A plot of DH vs $\log_{10}$ (protease amount) showed a linear relationship at each optimum temperature (data not shown). Activities of the three proteases were compared at their optimum temperatures (Table 3). Calculations were based on amount of each protease required for 50% hydrolysis. Optimase APL-440 and Alcalase 2.4L exhibited almost the same activity at 70°C. However, Optimase APL-440 showed slightly higher efficiency than Alcalase 2.4L based on its relatively lower cost. Prozyme 6 showed the lowest activity and efficiency. Based on these results, Optimase APL-440 was selected for further study. Furthermore, selection of Optimase APL-440 would be advantageous for industrial use since pasteurization occurs at high temperature.

Optimization of hydrolysis conditions

Responses of two dependent variables to hydrolysis conditions for Optimase APL-440 were followed (Table 4). Estimated regression coefficients for each dependent variable were obtained from these responses by multiple linear regression (Table 5). For amount of TSP, regression coefficients showed that pH (X_1), temperature (T, X_2), substrate concentration (S, X_4), and enzyme/substrate ratio (E/S, X_5) had linear effects, and pH and T had quadratic effects. Interactions on amount of TSP were observed between pH and T (p<0.001), T and S (p<0.10), and reaction time (t, X_3) and E/S (p<0.10). The largest value of estimated regression coefficient for S (b_4 = 0.54) indicated that it was the most important linear variable influencing amount of TSP. The positive value implied that amount of TSP increased with increasing S. Temperature was the second most important linear variable (b_2 = -0.34). The net effect of the linear and quadratic terms of pH and T suggested that amount of TSP increased until either pH or T reached an optimum point, following which the amount of TSP decreased as pH or T further increased. The best explanatory model equation for amount of TSP (Y_1) is as follows:

$$Y_1 = 2.14 - 0.26 \ X_1 - 0.34 \ X_2 + 0.54 \ X_4 + 0.09 \ X_5 - 0.23 \ X_1{}^*X_1 - 0.16 \ X_2{}^*X_2 - 0.25 \ X_1{}^*X_2 - 0.08 \ X_2{}^*X_4 + 0.09 \ X_3{}^*X_5$$

The pH, T, and E/S had linear effects on DH, and pH and T had quadratic effects. Interaction effect was significant between pH and T (p<0.001). Temperature was the most important linear variable affecting DH and had the highest regression coefficient (b_2 = -12.46), followed by pH (b_1 = -9.48). The best explanatory model equation for DH (Y_2) is as follows:

$$Y_2 = 62.40 - 9.48 \ X_1 - 12.46 \ X_2 + 2.62 \ X_5 - 8.16 \ X_1{}^*X_1 - 5.74 \ X_2{}^*X_2 - 8.74 \ X_1{}^*X_2$$

The model adequacy for each equation was tested by the coefficient of determination (R^2) and lack of fit. The model for amount of TSP (R^2 = 0.9696) indicated that only 3% of the
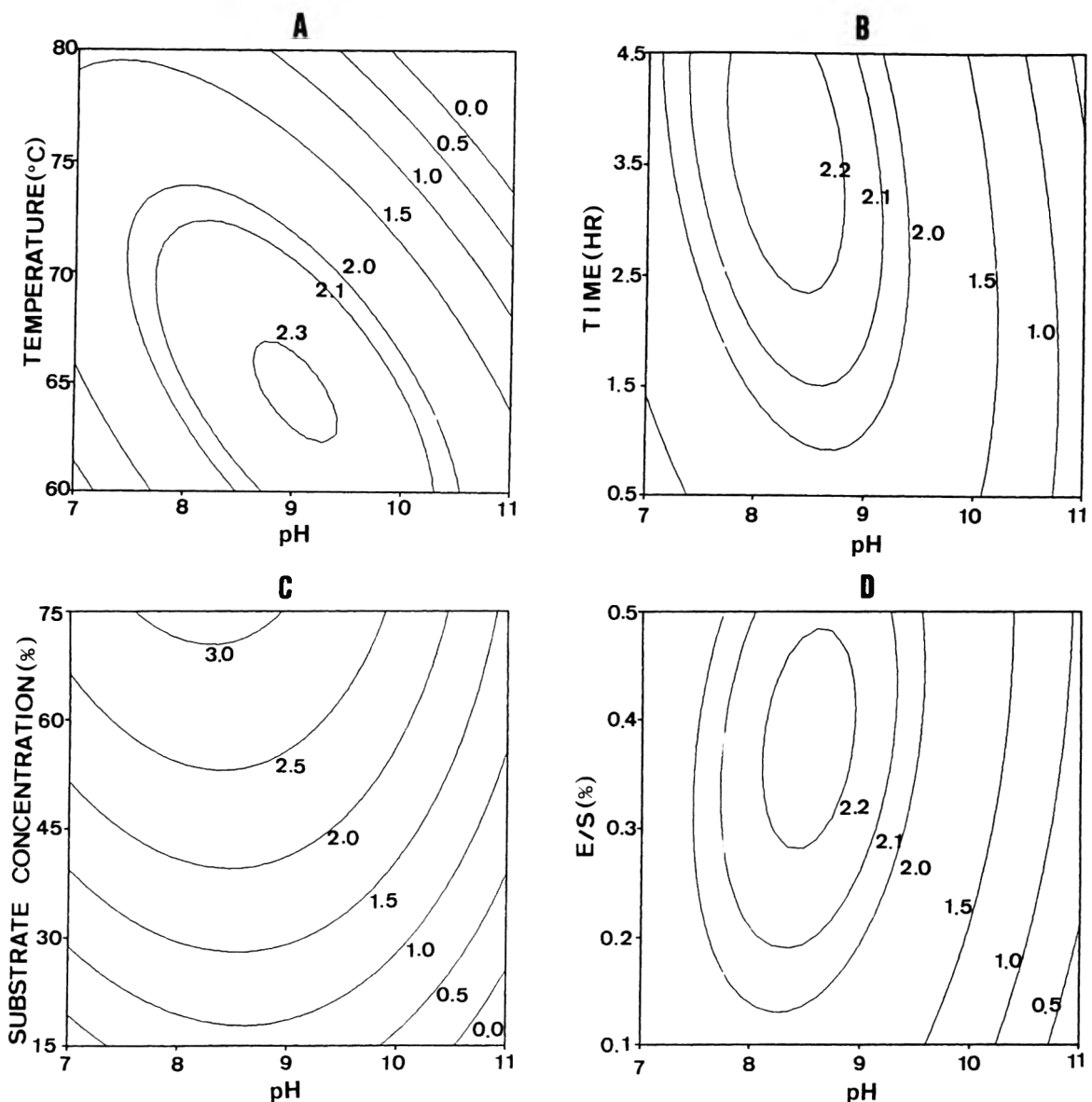
Fig. 5—Contour plots of effects of pH and temperature (A), reaction time (B), substrate concentration (C), and Optimase APL-440/ substrate ratio (D) on the amount of TSP.

total variation was not explained by the model. The coefficient of determination for DH was 0.9606, indicating that only 4% of the total variation was not explained by the model. These models were considered adequate with satisfactory R^2 values ($>$ 0.85) and significant F values. Lack of fit was significant, which means the order of regression equation was not secondary. Although the test of lack of fit was significant, high R^2 values indicated that the model was adequate.

Contour plots of the amount of TSP as a function of two different independent variables were developed (Fig. 5A–5D). Contours were plotted with the other three independent variables fixed at 0 coded levels.

Evaluation of effects of pH and temperature on the amount of TSP indicated that optimum pH was 9 and optimum temperature was 65°C (Fig. 5A). The temperature was slightly lower than that determined previously, indicating interactions between pH and temperature. Also, interaction effect was significant between pH and temperature ($p<0.001$). Other contour plots showed that pH optimum was between 8 and 9. This result suggested that pH adjustment was not needed during hydrolysis since the pH of both intrinsic CPBs and hydrolysate was between 8 and 9. Although pH drop occurred during hydrolysis, pH did not decrease below 8.0.

Effect of pH and reaction time on amount of TSP is shown (Fig. 5B). At higher pH (pH 10–11), amount of TSP was constant regardless of reaction time, indicating enzyme inactivation at high pH and 70°C (0 coded level). However, at optimum pH (pH 8–9), the amount of TSP increased as reaction time increased, and remained constant after 2.5 hr.

The amount of TSP increased with increasing substrate concentration (Fig. 5C). Optimum conditions were achieved at the highest substrate concentration [75% (w/v)] and pH 8–9. The greatest amount of TSP was obtained under these conditions. The effect of E/S on the amount of TSP production is shown (Fig. 5D). The amount of TSP increased with increasing E/S up to 0.3% (v/w) and thereafter remained constant regardless of increases in E/S.

Contour plots of DH as a function of two different independent variables are shown (Fig. 6A–6D). These were plotted for two independent variables as described previously. Highest DH was obtained at 65°C and near pH 9 (Fig. 6A). DH increased with increasing reaction time at pH 8–9 (Fig. 6B). Further, DH increased with increasing E/S up to 0.3% (v/w) of E/S (Fig. 6D). These optimum hydrolysis conditions were consistent with those from contour plots for amount of TSP. Interestingly, DH increased as substrate concentration decreased to 45% (w/v), suggesting that high DH did not coincide with a high amount
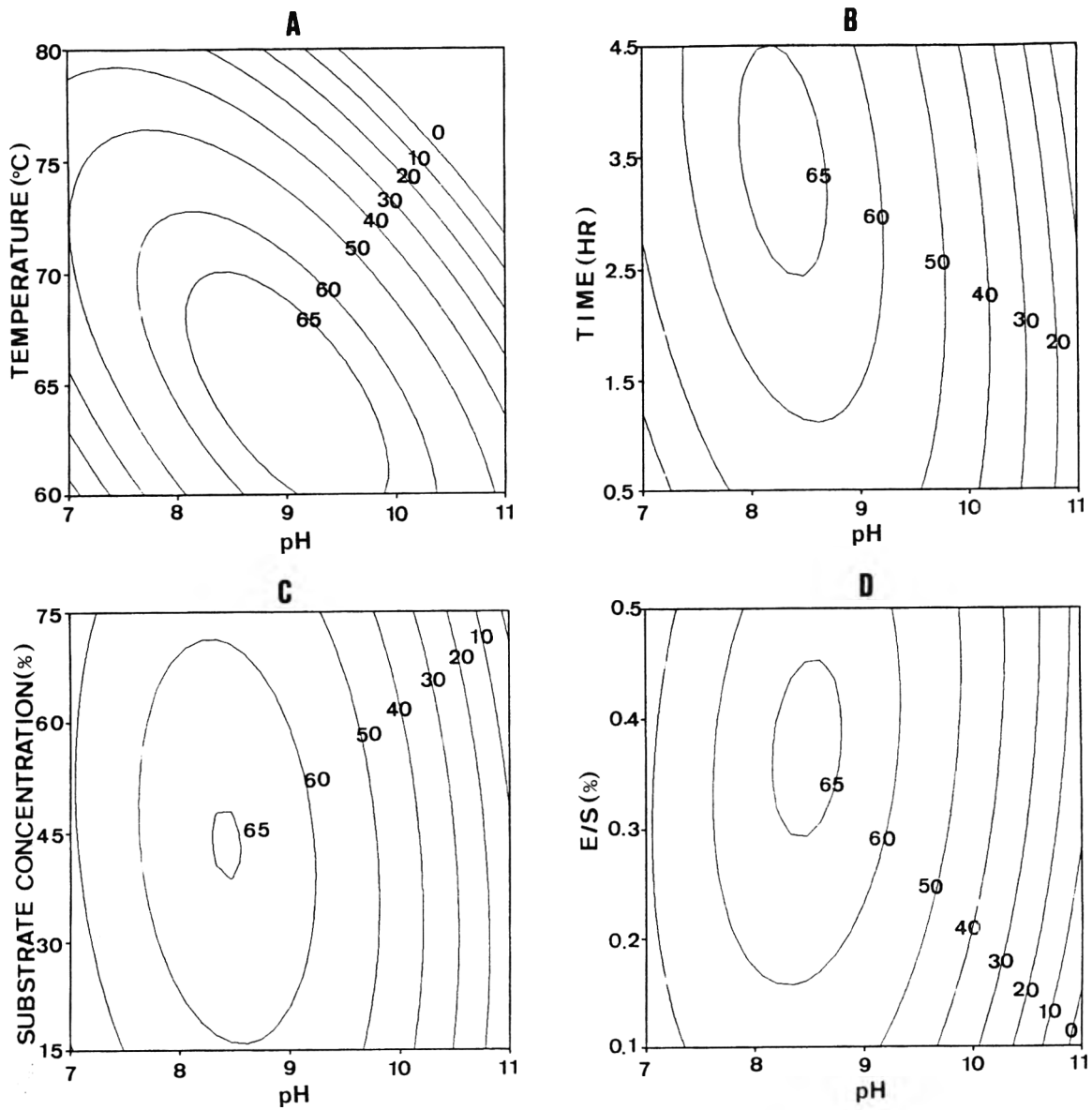
Fig. 6—Contour plots of effects of pH and temperature (A), reaction time (B), substrate concentration (C), and Optimase APL-440/ substrate ratio (D) on DH.

of hydrolysate (Fig. 6C) and amount of TSP may be a more reliable dependent variable. Surówka and Fik (1992), who measured the production of protein hydrolysate from chicken heads, reported that hydrolysis rate increased as amount of water increased (substrate concentration decreased) up to 75% of the raw material. Solubilization of lean beef tissue decreased markedly with increasing meat:liquid ratio (O'Meara and Munro, 1984b).

Based on these results, optimum hydrolysis conditions for enzymatic hydrolysis of CPBs with Optimase APL-440 were determined as pH 8–9, 65°C, 2.5 hr reaction time, 75% (w/v) substrate concentration, and 0.3% (v/w) Optimase APL-440. Since pH adjustment was not necessary, the highest amount of hydrolysate could be obtained by control of temperature (65°C) and reaction time (2.5 hr) with addition of 0.3% Optimase APL-440 to 75% (w/v) CPBs.

Hydrolysates produced from CPBs could serve as feedstocks for the production of value-added seafood flavor extracts. This may make it possible to utilize CPBs to their full potential and could greatly reduce the amount of solid waste discharged from crayfish processing plants.

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Ms received 11/23/94; revised 2/28/95; accepted 3/31/95

ᵞ Thermal Gelation of Pork, Beef, Fish, Chicken and Turkey Muscles as Affected by Heating Rate and pH

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ABSTRACT

Effects of heating rate (3°C or 0.7°C/min) and pH (5.5, 6.0, 6.5, or 7.0) on thermal gelation properties of different muscle systems were evaluated (10% protein, 2% NaCl) using pork, beef, fish, and chicken and turkey (breast and thigh) muscles. Results indicated that, at pH 6.5 and 7.0, force required to rupture the gel (Pf), force required to move plunger through the gel (Fp), and viscosity index (Ni), using a slow heating rate, were higher than with rapid heating. All muscles (except breast muscles with the slow heating rate) yielded higher (P<0.05) gel strength (Fp, Pf) at pH 6.0 than at the other pHs.

Key Words: thermal gelation, pork, beef, fish, chicken, turkey

INTRODUCTION

GELATION OF MUSCLE PROTEIN contributes to desirable texture and stabilization of fat and water in processed meat products. Formation of a gel with desirable texture is dependent not only on ionic strength, pH, heating temperature and rate, and post-mortem history of the muscle, but also on animal species and muscle type. The latter is a consequence of the intrinsic differences in myosin isoforms, initial pH, protein extractability, and functional properties of meat proteins in different muscle species or fiber types (Samejima et al., 1992).

Montejano et al. (1984) reported that heat-induced gels prepared from surimi, beef, pork and turkey did not differ when specimen deformation did not exceed 80% of original length. Amato et al. (1989) found that poultry thigh gels had higher shear stress and strain, and water retention values than breast gels. Turkey gels had higher stress than chicken gels when 2% NaCl was added. However, they did not determine whether the variance in pH was responsible for observed fracture trends.

It has been suggested that protein extractabilty increases with increasing pH (Richardson and Jones, 1987; Xiong and Brekke, 1991), and gelation properties are influenced by pH (Daum-Thunberg et al., 1992; Foegeding, 1987; Samejima et al., 1992). However, little information is available on the effect of pH on gelation properties of ground muscle systems.

Heating rate also influences thermal gelation properties. Camou et al. (1989) reported that gel strength of salt-soluble protein from pork semimembranosus muscle decreased as heating rate (0.28, 0.63 and 1.42°C/min) increased. Barbut and Mittal (1990) studied the effects of heating rates (0.31, 0.51, 1.22, and 1.62°C /min) on gelation, stability and texture of postrigor (beef) meat batters. Slower heating rates resulted in higher modulus of rigidity (G), hardness, gumminess and chewiness values and higher fluid losses during cooking. Foegeding et al. (1986) reported that a bovine myosin gel, formed by heating at a linear rate of 2°C/min from 20 to 70°C, was stronger than gel produced by 20 min of isothermal heating at 70°C. Xiong and Brekke (1991) later confirmed this finding. They indicated that fast heating (20 min at 70°C, which brought the sample temperature from 20 to 70°C within 5 min) resulted in weaker breast myofibril

gels than slow heating (linear heating rate at 1°C/min from 20 to 70°C).

Differences in protein functionality depend upon origin of species and fiber types (Amato et al., 1989; Xiong and Brekke, 1991; Samejima et al., 1992). Most studies have focused on myofibril systems which makes it very difficult to compare results to predict the differing functional behavior of any given ground muscle system between different species.

The objective of this study was to apply the compression deformation test to evaluate differences in thermal gelation properties of pork, beef, fish, chicken and turkey muscles on an equal protein basis with respect to the effects of heating rate and pH.

MATERIALS & METHODS

Muscle sample

Postrigor semimembranosus muscles of pork (n=4, commercial barrows and gilts) and beef (n=4, A maturity steers and heifers) were obtained at 24 hr postmortem from the University of Illinois Meat Science Laboratory. Twenty broilers (6 wk old) and three turkeys (6 mo old) were obtained from University of Illinois Poultry Farm and Sprinkle Turkey Farm, respectively, and slaughtered and chilled. Chicken breast (CB), chicken thigh (CT), turkey breast (TB) and turkey thigh (TT) were obtained after birds had been chilled in ice water (0°C) for 24 hr. Live catfish were obtained from a commercial farm. Catfish (n=40) were mechanically stunned, skinned, eviscerated, and fillets were removed. The fillets were put in plastic bags and chilled in ice water for 24 hr. Samples from each species were trimmed of fat and epimysial connective tissue when necessary, then pooled and homogenized (Model R6Y, Robot Coupe USA, Inc. Ridgeland, MS) in similar quantities (1000 g) for 1 min, vacuum-packaged (Model AGW Multivac, Koch Supplies Inc., Kansas City, MO), and stored at −80°C for subsequent analysis.

Chemical analyses

Five samples of each muscle system were analyzed for protein, using a macro-Kjeldahl procedure (AOAC, 1990), for moisture by weight loss of a 5-g sample dried at 105°C for 24 hr (AOAC, 1990), and for fat by repetitive extraction of moisture-free samples with an azeotropic mixture (87:13) of chloroform and methanol (Novakofski et al., 1989). Muscle pH was determined with a pH meter (Model 720A, Orion Research Incorporated, Boston, MA) fitted with a Ross sure-flow combination electrode (Model 81-72) on a 5-g sample homogenized with 25 mL distilled water. Percent free water was determined as a measure of water holding capacity (WHC) using the filter-press procedure described by Wierbicki and Deatherage (1958).

Preparation of heat-induced gels

Samples were adjusted to 10% protein and target pH levels (5.5, 6.0, 6.5, and 7.0) with 80 mL buffer solutions {1M 2-[N-morpholino]-ethanesulfonic acid (MES) and 5% NaCl} of different pH, varying amounts of distilled water and 1N HCl or NaOH. Based on a preliminary experiment, a series of buffer solutions of different pH were made to bring the different muscle system to the different target pHs. The final muscle slurry (200 mL) containing 2% NaCl and 0.4M MES at each pH was divided equally into eight aliquots and placed into plastic containers (4.8 cm high and 3.0 cm in diameter). Eight aliquots were heated isothermally in a 70°C water bath (at 3°C/min), the other eight heated at 0.7°C/min (initial temperature = 30°C) to a final internal temperature 70°C. Copper-constantan thermocouples (size 0.25 mm in diameter, Omega Engineering, Inc., Stamford, CT) attached to a recording ther-

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Table 1—Muscle characteristics[g]

Muscle species	pH	Protein (%)	Moisture (%)	Fat (%)	Free H$_2$O(%)	SSP (W%)	SSP (P%)	Collagen (mg/g)
Pork	5.60[d]	21.00[d]	74.98[d]	3.82[c]	34.02[a]	10.51[cd]	50.56[b]	6.49[b]
Beef	5.53[d]	22.29[c]	73.65[e]	3.93[c]	23.77[bc]	11.43[bc]	51.26[b]	4.49[c]
Fish	6.61[a]	17.50[g]	78.01[a]	4.81[b]	38.46[a]	9.10[d]	52.01[b]	6.79[b]
CB	6.19[b]	23.60[b]	75.61[c]	1.54[d]	13.47[d]	13.74[a]	58.22[b]	4.02[c]
CT	6.54[a]	19.80[f]	76.94[b]	3.79[c]	21.77[c]	13.85[a]	69.94[a]	8.89[a]
TB	5.87[c]	25.27[a]	73.64[e]	0.92[e]	31.14[ab]	12.83[ab]	50.73[b]	4.06[c]
TT	6.23[b]	20.36[e]	72.57[f]	7.11[a]	23.87[bc]	11.68[bc]	57.34[b]	8.29[a]
SEM	0.08	0.14	0.07	0.15	2.23	0.56	2.77	0.53

[a-f] Means in the same columns with different superscript differ (P < 0.05).

[g] Where: CB = chicken breast, CT = chicken thigh, TB = turkey breast, TT = turkey thigh. SSP(W%) = salt-soluble protein, percent of sample weight; SSP(P%) = salt-soluble protein, percent of total protein; SEM = standard error of means.

mometer (Model CR5 digital recorder, Campbell Scientific, Logan, UT) were inserted in the center of gel containers to monitor temperature during cooking. Cooked samples were immediately chilled at 4°C for 1 hr prior to gel strength measurement.

Gel strength

Gel strength was measured using an Instron Universal Testing Machine (Model 1122, Instron Corp., Park Ridge, IL). A 1.3-cm-diameter flat ended plunger was used to compress the heat-induced gels to penetrate 60% original height. A compression load cell setting of 1-2 kg, a crosshead speed of 100 mm/min, and a chart speed of 100 mm/min were used. The rheological parameters of the muscle gels ($\approx$ 4°C) were measured from force-deformation curves using the methods described by Hickson et al. (1982). The force required to penetrate or rupture the gel (Pf) and the force required to move the plunger through the gel (Fp) were determined from the force-deformation curve, and viscosity index (Ni) and apparent elasticity (Ea) were calculated. Force units (expressed as grams) may be converted to dynes by multiplying acceleration (dyne = g $\times$ 9.8 cm/s^2 = 10^{-5} Newton). Viscosity index units may be converted to poise by the factor 5.88 $\times$ 10^6.

Cooking loss

After measurement of gel strength, gel samples were then used for the cooking loss evaluation as described by Samejima et al. (1969). Compressed gels were centrifuged at 5,000 $\times$ g for 15 min. Cooking loss of the gels was calculated as [(slurry weight before heating $-$ gel weight after centrifugation)/(slurry weight before heating)] $\times$ 100.

Salt-soluble protein (SSP)

SSP concentration was determined on duplicate 10-g ground samples homogenized in 150 mL of 2% NaCl and 0.1 M MES (pH 6.0) cold solution ($\approx$ 4°C). Sample solutions were adjusted to pH 6.0 with 1 N HCl and 1 N NaOH and centrifuged at 4,000 $\times$ g for 10 min at 2°C, then decanted through Whatman #4 filter paper. Protein concentration of the SSP extract was determined using the Kjeldahl procedure (AOAC, 1990). SSP concentration was expressed as percentage of sample weight and as percentage of total protein.

Total collagen content

Collagen (based on hydroxyproline content) was determined for each sample using the modified procedure of AOAC (#990.26, AOAC, 1990). Each sample ($\approx$ 2g) was hydrolyzed in 10 mL 6N HCl at 105°C for 16 hr. The hydrolyzed solution was diluted with distilled water to 15 mL, then decolorized by filtering through activated charcoal and Whatman #2 filter paper. Duplicate 0.02-mL aliquots were diluted to 2 mL with distilled water (2 mL H$_2$O for blanks). Sample color and hydroxyproline standards were evaluated using a spectrophotometer (Beckman DU 640, Beckman Instruments, Inc., Fullerton, CA). Hydroxyproline content was extrapolated from the standard curve and converted to collagen content using the following formula: collagen (mg/g meat) = hydroxyproline (mg/g meat) $\times$ 8.

SDS-polyacrylamide gel electrophoresis (SDS-PAGE)

The muscle and salt-soluble protein samples were analyzed by SDS-PAGE described by Fritz et al. (1989). The stacking gel was 5% and the resolving gel 10% acrylamide.

Statistical analyses

A 4 $\times$ 2 $\times$ 7 factorial design was used to investigate the effects of pH (5.5, 6.0, 6.5, and 7.0), heating rate (fast and slow), and seven species and muscle types on muscle protein gelation properties; eight replicates were included in each treatment. Results were analyzed using the GLM procedures of SAS Institute, Inc. (1993). Analysis of variance was used to evaluate the effects of muscle species, heating rate, pH levels, and interactions. Least-square means and probability of difference procedures were used to separate means. Regression analysis was used to test for linear and quadratic response with pH as the independent variable and gel forming characteristics as dependent variables.

RESULTS & DISCUSSION

MUSCLE CHARACTERISTICS of the different species were compared (Table 1). There were wide ranges in chemical characteristics: ultimate pH, protein, moisture, and lipid content. Results suggest that CB had the highest (P<0.05) water holding capacity (lowest percent free water); however, TB had the highest protein content and the lowest lipid content (P<0.05). Fish muscle had the lowest protein and highest moisture, while it had lower water-holding capacity (WHC) and higher ultimate pH than most other species. It appears that the high ultimate pH of fish did not result in a high WHC. This may be related to the moisture-to-protein ratio. It may also be related to specific type of myosin. Salt-soluble protein (SSP) content as a percentage of sample weight was highest for CB and CT (P<0.05), numerically lowest for fish muscle. The SSP, as percent of total protein, was similar for most species except CT which had a high solubility.

The high ultimate pH (6.54) of CT may contribute to the high solubility due to less protein denaturation. Collagen is the major constituent of connective tissue. Thigh muscles from both chicken and turkey had the highest collagen content, and breast muscles the lowest (P<0.05). Amato et al. (1989) reported that collagen content of chicken breast and thigh, turkey breast and thigh was 4.68, 10.83, 4.62, and 5.36 mg/g meat, respectively. Collagen contents of chicken breast (4.02) and turkey breast (4.06) found in our study were consistent with reported values, while the disparity in collagen contents of thigh muscles between the two studies may be attributed to differences in trimming of epimysial connective tissue prior to sample preparation.

Analysis of variance (Table 2) demonstrated that species and pH had significant effects on all gel characteristics. Heating rate affected all gel properties except cooking loss and elasticity (Ea). The pH had quadratic effects on cooking loss and elasticity (Ea), and cubic effects on Fp, Pf, and Ni. There were no interactions among species $\times$ pH $\times$ heating rate on gel strength characteristics except cooking loss. Significant interactions occurred for species $\times$ pH on all characteristics, and for pH $\times$ heating rate on most characteristics, therefore data were not pooled across heating rate, pH, and species.

Gel strength (Fp, Pf, Ni, and Ea) was quantified using muscle samples adjusted to 10% protein and the indicated pH for both heating rates (Fig. 1, 2, 3, and 4). Gels produced using the fast heating rate and pH 6.0 had the highest Fp and Pf for all species
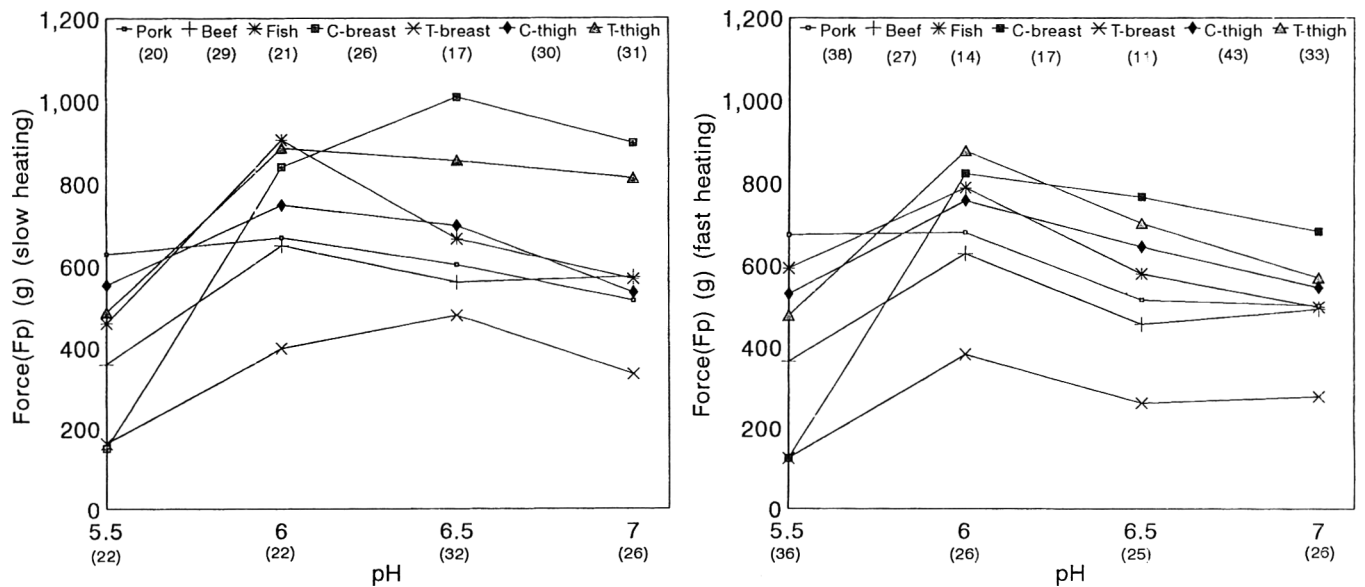
Fig. 1—Force required to move plunger through muscle gels (Fp). Means of eight replicates. Gelation conditions: 10% protein, 2% NaCl, 0.4M MES at various pHs; heating from 30 to 70°C at 0.7°C/min (slow heating) or isothermally in 70°C at 3°C/min (fast heating). Values in parenthesis are standard error of means (SEM) for comparisons across pH for a given species or among species at a given pH.
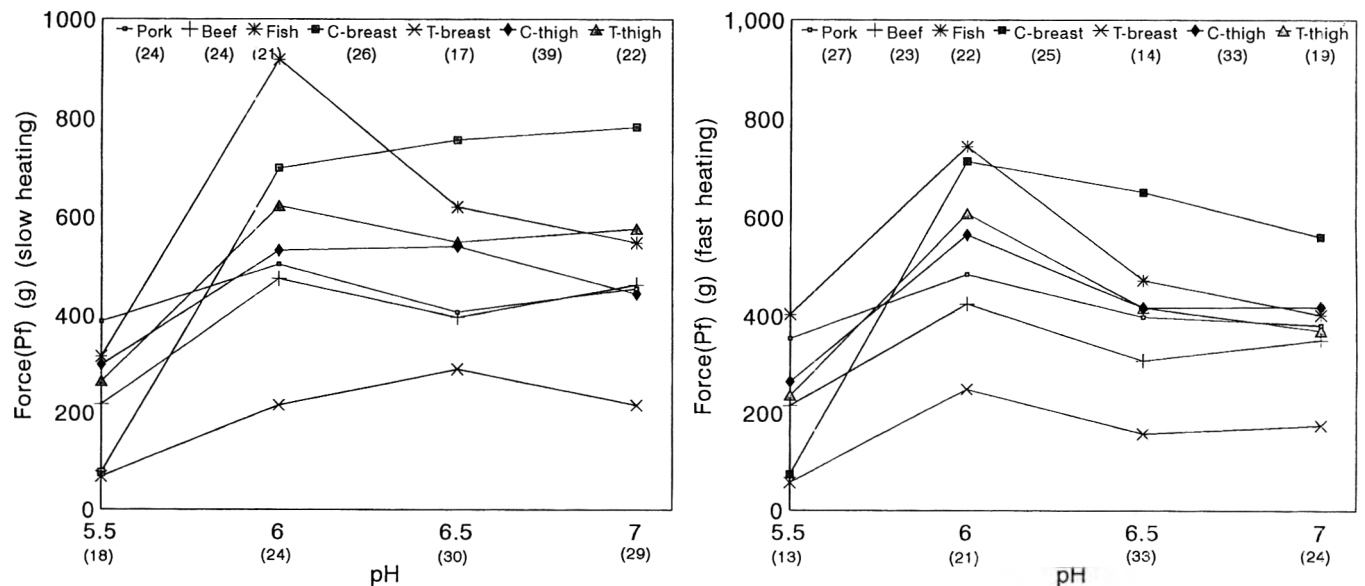
Fig. 2—Force required to rupture muscle gels (Pf). Means of eight replicates. Gelation conditions: 10% protein, 2% NaCl, 0.4M MES at various pHs; heating from 30 to 70°C at 0.7°C/min (slow heating) or isothermally in 70°C at 3°C/min (fast heating). Values in parenthesis are SEM for comparisons across pH for a given species or among species at a given pH.

Table 2—ANOVA of muscle gel characteristics (probability values)[a]

Traits	Main effects			Interaction					
	Species	pH	HR	Sp×HR	Sp×pH	pH×HR	Sp×pH×HR	pH^2	pH^3
Fp (g)	0.0001	0.0001	0.0001	0.025	0.0001	0.0001	0.22	0.0001	0.0001
Pf (g)	0.0001	0.0001	0.0001	0.47	0.0001	0.0001	0.31	0.0001	0.0001
Ni (×10^{-3}) (g·min/mm^2)	0.0001	0.0001	0.0009	0.04	0.0001	0.0001	0.21	0.0001	0.0001
Ea (g/mm·min)	0.01	0.0001	0.06	0.27	0.001	0.03	0.79	0.0001	0.65
Cooking loss (%)	0.0001	0.0001	0.50	0.02	0.0001	0.80	0.03	0.0001	0.19

[a] Where: Fp = force to move plunger through gel. Pf = force to rupture gel. Ni = viscosity index. Ea = elasticity. SP = species. HR = heating rate. Species = seven species and muscle types.

(Fig. 1 and 2); however, these produced using the slow heating rate resulted in the highest Fp and Pf at pH 6.0 for pork, beef, fish, and TT samples (P<0.05). TB had the lowest Fp and Pf (P<0.05) at pH 6.0, 6.5, and 7.0 for both heating rates, while CB had the highest Fp and Pf (P<0.05) at pH 6.5 and 7.0. At pH 5.5, pork had the highest Fp while TB and CB had the lowest

Fp and Pf (P<0.05) compared to the other species. In contrast, at pH 5.5 cooking loss was higher for pork and lower for CB and TB (P<0.05). A cubic effect (P<0.0001) for Fp and Pf was observed with pH. The data suggest that Fp was markedly pH-dependent with optimal binding between pH 6.0 and 6.5 for all species studied.
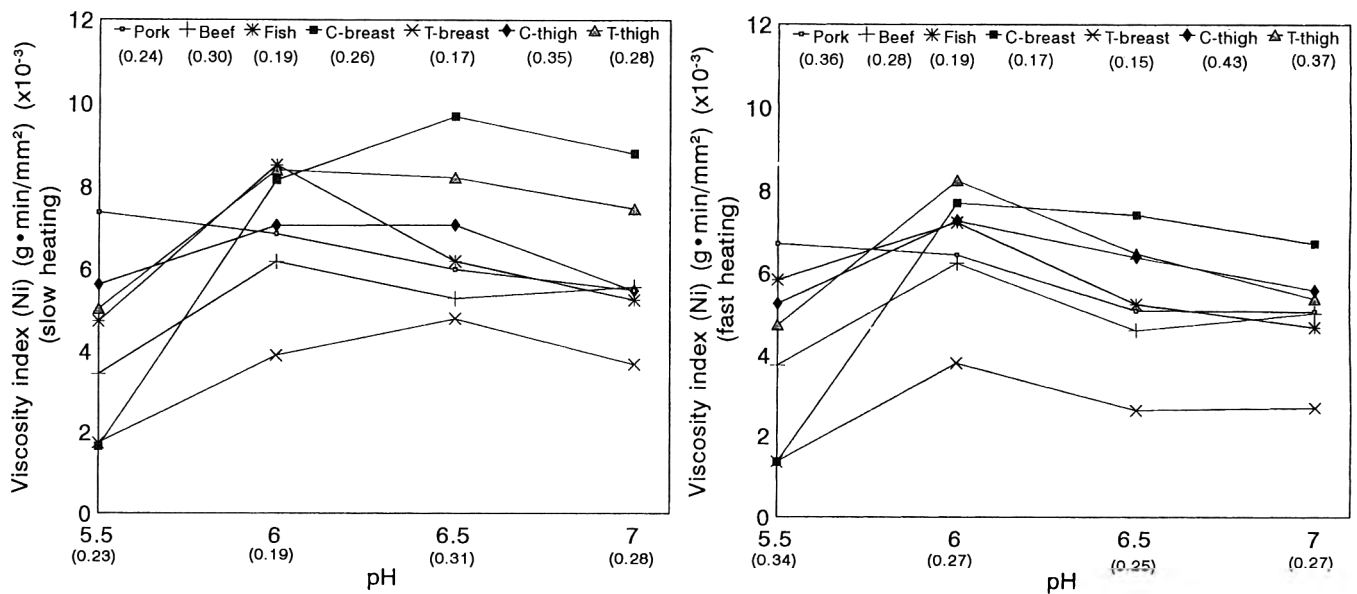
Fig. 3—Viscosity index of muscle gels (Ni). Means of eight replicates. Gelation conditions: 10% protein, 2% NaCl, 0.4M MES at various pHs; heating from 30 to 70°C at 0.7°C/min (slow heating) or isothermally in 70°C at 3°C/min (fast heating). Values in parenthesis are SEM for comparisons across pH for a given species or among species at a given pH.
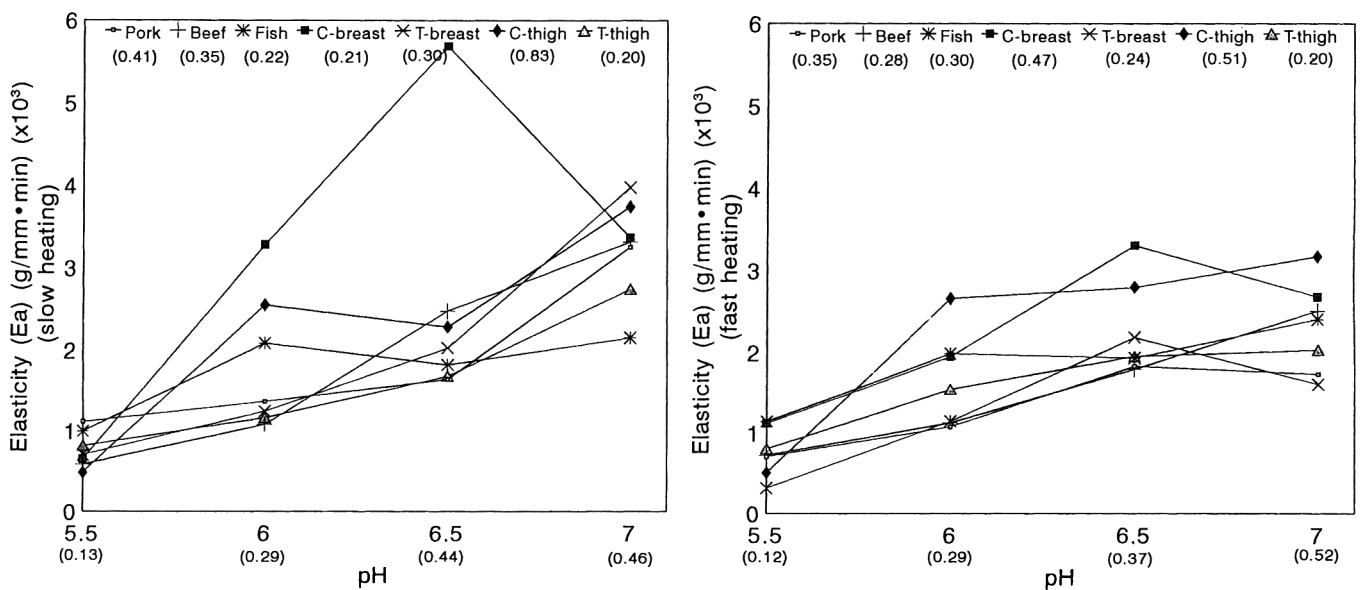
Fig. 4—Elasticity of muscle gels (Ea). Means of eight replicates. Gelation conditions: 10% protein, 2% NaCl, 0.4M MES at various pHs; heating from 30 to 70°C at 0.7°C/min (slow heating) or isothermally in 70°C at 3°C/min (fast heating). Values in parenthesis are SEM for comparisons across pH for a given species or among species at a given pH.

Estimated viscosity index (Ni) (Fig. 3) showed at almost all pHs, slow heating rates resulted in higher viscosity values than did fast heating rates. This was particularly true at pH 6.5 where viscosity of slow heating rates were higher (P<0.05) than those of fast heating rates for all species except beef and CT. The fast heating rate at pH 6.0 produced the highest (P<0.05) viscosity for all species except pork (Ni) which decreased as pH increased from 5.5 to 7.0. However, slow heating at pH 6.5 resulted in the highest (P<0.05) viscosity for CB and TB. In parallel with Fp and Pf profiles among all species, TB had the lowest (P<0.05) viscosity at pH > 5.5, while CB had the highest viscosity (P<0.05) at pH 6.5 and 7.0. Similar to Fp curves at pH 5.5, CB and TB had the lowest (P<0.05) while pork had the highest viscosity among all species. The pH had a cubic effect (P<0.0001) on viscosity. These data indicate that viscosity was pH-dependent with higher values at pH 6.0 and 6.5 for most species and muscle types. Xiong and Brekke (1989) reported that chicken breast myofibrils exhibited a greater apparent vis-

cosity than leg myofibrils. Asghar et al. (1984) and Morita et al. (1987) noted a greater viscosity of chicken breast myosin than leg myosin within the pH range 5.4 to 6.0. They suggested that the polymorphism of myosin and the ability of white myosin to form longer filaments than red myosin might explain viscosity differences. However, our results from ground muscle systems were not consistent with those reported in myofibril or myosin studies.

For calculated elasticity values (Fig. 4), CB had higher values (P<0.05) at pH 6.5 with both heating rates than the other species. At pH 5.5, all species had similar elasticities; differences in elasticity became larger as pH increased. The pH had a quadratic effect on gel elasticity (P<0.0001).

Cooking loss was quantified using muscle samples adjusted to 10% protein and the indicated pH for both fast and slow heating rates (Fig. 5). Fast heating rates brought the internal sample temperature from 22 to 70°C within about 16 min (3°C/min), while the slow heating rates required ≈ 68 min to bring
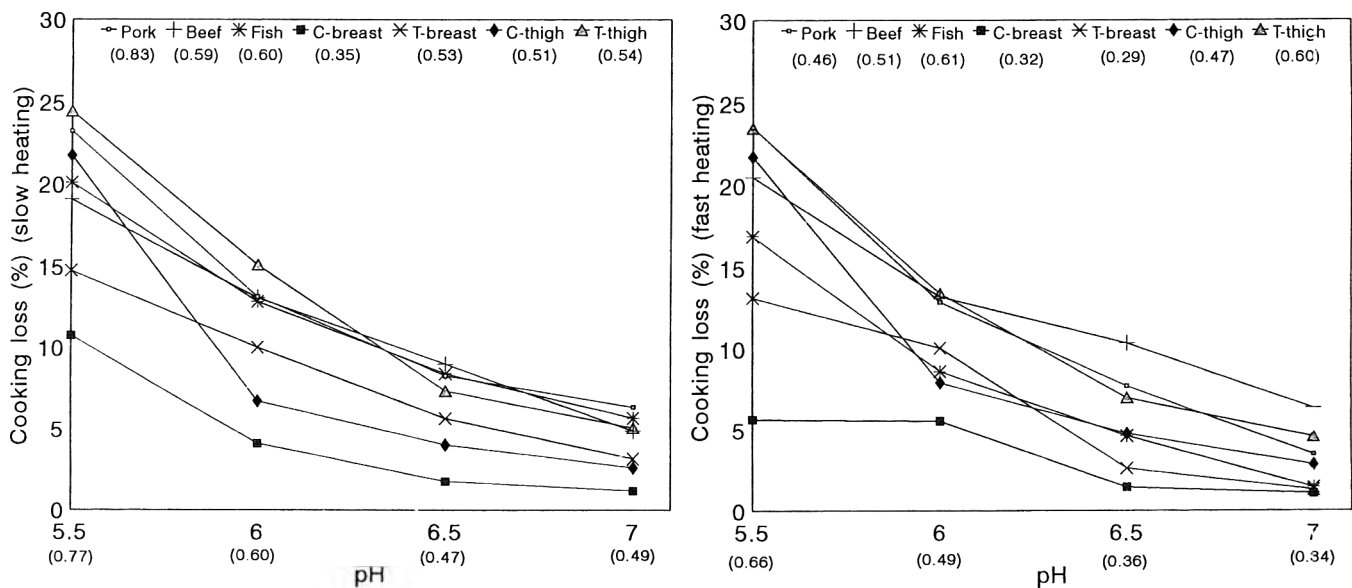
Fig. 5—Cooking loss of muscle gels. Means of eight replicates. Gelation conditions: 10% protein, 2% NaCl, 0.4M MES at various pHs; heating from 30 to 70°C at 0.7°C/min (slow heating) or isothermally in 70°C at 3°C/min (fast heating). Values in parenthesis are SEM for comparisons across pH for a given species or among species at a given pH.

the internal temperature to 70°C (0.7°C/min). Cooking losses decreased with increasing pH (P<0.05). The pH had a quadratic effect (P<0.0001) on cooking loss.

The consistent effect of pH on cooking loss indicates that WHC of muscle protein was very dependent on pH with all species responding similarly to pH changes. This suggests an increase in electrostatic repulsion among protein molecules and an increase in protein-solvent interactions as pH increased from 5.5 to 7.0. The increased protein-solvent interactions could increase the solubility of protein, thus improve water retention. Among different species, chicken breast had the lowest (P< 0.05) cooking loss at each pH for both heating rates, consistent with its lowest free water percentage. However, beef, pork and TT prepared using the two heating rates had higher cooking loss at most pH levels than the other species. Cooking loss of TB muscle was not a direct reflection of its very low gel strength, although the quantity of water added to TB to adjust the protein to 10% was the highest of all species.

Overall results indicated that gels from CB and CT had the highest strength and cooking yield, while beef and pork produced weaker gels and higher cooking losses. The variation in gelation properties among species did not appear to be related to muscle fiber types. These differences may be related to the difference in postmortem initial and/or ultimate muscle pH. Lan et al. (1995) reported that DFD muscle with initial ultimate pH of 7.0 had lower cooking loss and higher gel strength (Fp, Pf, Ni) at all adjusted pHs (5.5, 6.0, 6.5, and 7.0) than normal pork muscle with initial ultimate pH 5.5. They suggested that muscle with lower initial pH may undergo more protein denaturation which would reduce protein functionality and sensitivity to pH.

However, this trend was not so obvious on the gelation properties of fish and TT with respect to their high initial pH. This may be due to the high fat content in muscle tissues, and for fish muscle, this may also be related to its high moisture-to-protein ratio. Amato et al. (1989) reported that thigh gels had higher shear stress, shear strain, and water retention values than breast gels. Turkey gels had higher stress than chicken gels. In their study, pH 6.07 was observed for both chicken and turkey thigh, and 5.54 and 5.64 for chicken and turkey breast, respectively. They suggested that differences in stress values between muscle groups might be due to inherent pH differences. Our results also suggest that the breast muscles used in their study were not at optimum pH (6.5) for gelation.

Overall results also demonstrated that slow heating rates produced higher Fp, Pf, and Ni and higher cooking losses than fast heating rates for all but two (beef and CT) species at pH 6.5 and 7.0. This was in agreement with observations of Barbut and Mittal (1990), Foegeding et al. (1986), and Northcutt et al. (1993). They suggested that slower heating rates would allow more time for proteins to unfold and interact. Our results also agreed with those of Hermansson (1978) who suggested that denaturation of proteins prior to aggregation (related to slow heating) resulted in a finer gel structure and greater gel strength than if random aggregation occurred simultaneously with or prior to denaturation. Camou et al. (1989) reported that protein loss in the expelled water, after compression, was lower for slower heating rates. Reducing protein loss by slow heating may contribute to stronger gels. Although varying markedly, collagen content of muscles did not appear to be related to gel strength when comparing its content and gel strength of muscle. This may be due to the low collagen content in the 10% muscle protein gel matrices. Bailey and Light (1989) suggested that in cooked muscle only 10–15% of collagen is solubilized. Salt-soluble protein is largely responsible for textural properties of processed meat (Asghar et al., 1985; Li-Chan et al., 1985), and solubility differences have also been reported between fiber types (Xiong and Brekke, 1991; Samejima et al., 1992). Previous work in our lab (Lan et al., 1993) indicated that pH 6.0 yielded the highest percentage of SSP for beef longissimus dorsi and semimembranosus muscles; thus, in our current study, protein solubility of different species and muscle type were measured at pH 6.0. The solubility values did not appear to be related to gel strength and cooking loss. Electrophoretic analysis suggested no appreciable differences in muscle protein composition or salt-soluble protein composition of different species (SDS-PAGE data not shown).

CONCLUSIONS

DIFFERENCES EXIST in gelation properties between species and muscle types on an equal protein basis. Chicken breast and thigh had the highest gel strength and water binding ability for both slow and rapid heating rates at pH>6.0, and turkey breast had the lowest gel strength. Muscle gelation properties were pH-dependent with highest gel strength occurring at pH 6.0 to 6.5, whereas cooking loss decreased as pH increased. Results suggest

Thermal Gelation of Myofibrils from Pork, Beef, Fish, Chicken and Turkey

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ABSTRACT

Myofibril gels with 5, 7, or 10% protein and 2% NaCl were prepared from pork, beef, fish, chicken and turkey muscles using a heating rate of 0.7°C/min and pH 6.0 to compare the thermal gelation properties of myofibrillar protein from different species. The force required to rupture gel (Pf), force required to move plunger through gel (Fp), viscosity index (Ni), and elasticity (Ea) increased and cooking loss decreased with increasing protein contents. Myofibrils of chicken breast and thigh had lower and fish and turkey thigh had higher cooking loss at protein contents of 7% or 10%.

Key Words: myofibril gelation, pork, beef, fish, chicken, turkey

INTRODUCTION

THERMAL GELATION of myofibrillar protein is largely responsible for the textural properties of processed meat products (Asghar et al., 1985). Reported studies have identified large variations in functional properties of myofibrillar proteins associated with muscle fiber types. Park (1991) found that myofibrils from beef or pork formed gels with greater hardness than commercial fish surimi above 55°C. As concentration of myofibrillar protein in the gel increased, gel hardness increased regardless of cooking temperature or species. Samejima et al. (1992) reported that pork cardiac myofibrils always exhibited lower solubility and formed much weaker heat-induced gels than those produced by skeletal myofibrils under identical conditions of temperature, pH, ionic strength and protein content. Xiong and Brekke (1989, 1991) observed increased gelling ability of chicken breast myofibrils comapred with chicken leg myofibrils at protein concentrations < 3%. However, under similar gelling conditions, at 8% protein, Lavelle and Foegeding (1993) reported that turkey breast and thigh myofibril gels had similar rheological properties. Such conflicting results suggest that the mechanisms for gelation may be altered with varying protein concentration, Furthermore, most myofibril gelation studies have been conducted with rather dilute protein concentrations (0.1–3%), while in practice most processed meat products contain higher concentrations of protein. Research is needed to characterize protein gelling conditions so that they are more representative of the protein environments present during meat processing.

Species differences in gelation properties have been reported among pork, beef, fish, chicken and turkey breast and thigh muscles (Lan et al., 1995). Our objective was to determine whether such differences in muscle gelation are due to quantitative differences in myofibrillar, sarcoplasmic and stromal protein, or due to differences in binding ability of myofibrillar proteins among species.

MATERIALS & METHODS

Muscle sample preparation

Postrigor semimembranosus muscles of pork (n = 4, commercial barrows and gilts) and beef (n = 4, A maturity steers and heifers) were

obtained 24 hr postmortem from the University of Illinois Meat Science Laboratory. Twenty broilers (6 wk old) and three turkeys (6 mo old) were obtained from the University of Illinois Poultry Farm and Sprinkle Turkey Farm, respectively, and slaughtered and chilled. Chicken breast (CB), chicken thigh (CT), turkey breast (TB) and turkey thigh (TT) were obtained after birds had been chilled in ice water (0°C) for 24 hr. Live catfish were obtained from a commercial farm. Catfish (n = 40) were mechanically stunned, skinned, eviscerated, and fillets were removed. The fillets were put in platic bags and chilled in ice water for 24 hr. Samples from each species were trimmed of fat and epimysial connective tissue as necessary, then pooled and homogenized (Model R6Y, Robot Coupe USA, Inc., Ridgeland, MS) in similar quantities (1000g) for 1 min, vacuum-packaged (Model AGW Multivac, Koch Supplies Inc., Kansas City, MO), and stored at −80°C for subsequent analysis.

Preparation of myofibrillar, sarcoplasmic, and stromal protein fractions

Sarcoplasmic and myofibrillar proteins were prepared as described by Eisele and Brekke (1981). Homogenized muscle samples (400 g) were thawed and blendec in a Waring Blendor in 1600 mL buffer solution (0.05M NaCl, 0.05M potassium-phosphate, 5 mM EDTA, pH 7.0) for 4 min at maximum speed in a 2–4°C cold room. The suspension was mixed with a propeller (Model 106, Talboys Instrument Corp., Emerson, NJ) at 30 rpm for 4 hr followed by filtration through a strainer at 4°C. Connective tissue accumulated on the propeller shaft and on the household strainer was collected. The suspension was centrifuged at 7,000 x g for 30 min. The supernatant (sarcoplasmic protein) was retained. The resulting pellet was resuspended in 1600 mL buffer four times, each followed by centrifugation at 7,000 x g for 30 min at 2–4°C. Lipid accumulation at the top of the bottles during each centrifugation was discarded. Protein concentration of the final myofibril pellet, connective tissue residue, supernatant of sarcoplasmic protein fraction, and homogenized muscle tissue was determined using Kjeldahl nitrogen determination (AOAC, 1990). The concentration of each protein fraction as a percentage of total muscle protein was calculated based on the pellet and residue weight or supernatant volume and Kjeldahl nitrogen. For example: concentration of myofibrils as a percentage of total muscle protein = (protein concentration of myofibril pellet × weight of myofibril pellet)/(weight of total muscle protein). The myofibril pellets were stored at 4°C and evaluated within 24 hr of isolation.

Preparation of heat-induced gels

Myofibril samples were adjusted to the appropriate protein concentrations and pH 6.0 using 80 mL buffer solution containing 5% NaCl and 1M MES (2-[N-morpholino] ethanesulfonic acid), varying amounts of distilled water and 1N HCl and NaOH. The pH was measured with a pH meter (Model 720A, Orion Research Incorporated, Boston, MA) fitted with a Ross sure-flow combination electrode (Model 81–72). All gel samples contained 2% NaCl and 0.4M MES. A 200-g gel sample was divided into 8 plastic containers (4.8 cm high and 3.0 cm in diameter) for each protein level, then heated from 30°C to 70°C at 0.7°C/min in a water bath (Model 183, Precision Scientific Incorporated, Chicago, IL). Copper-constantoan thermocouples (0.25 mm in diameter, Omega Engineering, Inc. Stamford, CT) attached to a recording thermometer (Model CR5 digital recorder, Campbell Scientific, Logan, UT) were used to monitor temperature. Samples were chilled at 4°C for 1 hr prior to gel strength measurement.

Gel strength

Gel strength was measured using an Instron Universal Testing Machine (Model 1122, Instron Corp., Park Ridge, IL). A 1.3-cm-diameter

Table 1—Protein components and contents of muscles[g]

Species	Protein (%)	Collagen (%)	Sarcop (%)	Myofib (%)	Stromal (%)	Calculated protein[h] (%)
Pork	21.00[d]	0.65[b]	8.82	11.41	0.84	21.07
Beef	22.29[c]	0.45[c]	9.10	12.65	0.85	22.60
Fish	17.50[g]	0.68[b]	5.50	11.31	0.52	17.33
CB	23.60[b]	0.40[c]	9.46	12.57	0.32	22.35
CT	19.80[f]	0.89[a]	7.89	10.87	1.07	19.83
TB	25.27[a]	0.41[c]	9.71	13.01	0.41	23.13
TT	20.36[e]	0.83[a]	6.58	11.85	1.26	19.69
SEM	0.14	0.05				

[a-f] Means in same column with different superscripts differ (P < 0.05).

[g] Where: Sarcop = sarcoplasmic protein, Myofib = myofibril protein, Stromal = stromal protein, CB = chicken breast, CT = chicken thigh, TB = turkey breast, TT = turkey thigh, SEM = standard error of means.

[h] Calculated protein = Sarcop + Myofib + Stromal.

Table 2—ANOVA of myofibril gel characteristics (probability values)[a]

	Main effects		Interaction	Quadratic
Traits	species	protein	species × protein	protein
Fp (g)	0.0001	0.0001	0.0001	0.0001
Pf (g)	0.0001	0.0001	0.0001	0.0001
Ni (×10⁻³) (g·min/ mm²)	0.0001	0.0187	0.0001	0.0001
Ea (g/mm· min)	0.0001	0.0001	0.0180	0.5109
Cooking loss (%)	0.0001	0.0001	0.0001	0.0001

[a] Where: Fp = Force to move plunger through gel; Pf = Force to rupture gel; Ni = Viscosity index, Ea = elasticity, Protein: 5, 7, and 10%. Species = 7 myofibrils from different species and muscle types.

plunger was used to penetrate the heat-induced gels to deform 60% of original height along the long axis. A compression load cell setting of 1-2 kg, a crosshead descent speed of 100 mm/min, and a chart speed of 100 mm/min were used. The rheological parameters of the muscle gels ($\approx$4°C) were measured from force-deformation curves using the methods described by Hickson et al. (1982). Force required to penetrate or rupture the gel (Pf) and force required to move the plunger through the gel (Fp) were determined from the force-deformation curve, and viscosity index (Ni) and apparent elasticity (Ea) were calculated.

Cooking loss measurement

After gel strength measurement, the heated samples were used for cooking loss determination as described by Samejima et al. (1969). Compressed gel was centrifuged at 5,000 × g for 15 min. Cooking loss was calculated as [(slurry weight before heating – gel weight after centrifugation)/(slurry weight before heating)] × 100.

SDS-polyacrylamide gel electrophoresis (SDS-PAGE)

The myofibrillar and sarcoplasmic protein fractions were subjected to SDS-PAGE as described by Fritz et al. (1989). The stacking gel was 5% and the resolving gel 10% acrylamide.

Total collagen content

Collagen (based on hydroxyproline content) was determined for each muscle and myofibril sample using the modified procedure of AOAC (#990.26, AOAC, 1990). Five replicates of $\approx$ 2g muscle or 4g myofibril sample were hydrolyzed in 10 mL of 6N HCl at 105°C for 16 hr. The hydrolyzed solutions were diluted with distilled water to 15 mL and decolorized by filtering through activated charcoal and Whatman #2 filter paper. A 0.02-mL aliquot was diluted to 2 mL with distilled water in duplicate (2 mL H_2O for blanks). Absorbance at 560 nm of samples and hydroxyproline standards was determined using a spectrophotometer (Beckman DU 640, Beckman Instruments, Inc., Fullerton, CA). Hydroxyproline content was extrapolated from the standard curve and converted to collagen content using the following formula: collagen (mg/g sample) = hydroxyproline (mg/g sample) × 8.

Statistical analysis

A 3 × 7 factorial design was used to investigate the effect of protein concentrations (5, 7, or 10%) and species and muscle types on gelation properties of myofibril systems. Eight replications were used for each treatment. Data were analyzed using the GLM procedures of SAS Institute, Inc., (1993). Analysis of variance was used to evaluate the effects of muscle species, protein contents, and interactions. Least-square means and probability of difference procedures were used to separate means. Regression analysis was used to test for linear and quadratic response with protein concentration as independent variable and gel forming characteristics as dependent variables.

RESULTS & DISCUSSION

MYOFIBRILLAR, SARCOPLASMIC, AND STROMAL PROTEIN concentrations in muscle tissue of each species and muscle type determined in our study were compared (Table 1). Myofibrillar protein ranged from 51% for TB to 64% for fish. Sarcoplasmic protein ranged from 31% for fish to 42% for pork. Stromal protein contributed a very small percentage to the total protein ranging from 1.26% for TT to 0.32% for CB. These values were relatively consistent with the collagen content, suggesting that collagen is the predominant stromal protein. The fact that some of the stromal protein values determined were equal to or lower than the collagen values may be due to relatively large variations in sampling. The values of myofibrillar and sarcoplasmic protein concentrations of muscle tissues were generally consistent with a previous report that myofibrillar and sarcoplasmic proteins comprise $\approx$ 60% and 30% of the total muscle protein, respectively (Lawrie, 1991). Our low stromal protein values compared to the values from that report ($\approx$ 10%) were likely due to trimming of epimysial connective tissue during sample preparation. The calculated total protein values of these three protein fractions were similar to the total protein content. Fish, CT, and TT had lowest (P < 0.05) total percent protein among all species. Collagen concentrations were highest (P < 0.05) for CT and TT. Although varying markedly, the quantitative differences in myofibrillar, sarcoplasmic and stromal proteins between species did not appear to be related to differences in gelation properties of ground muscle tissue between species from our previous study (Lan et al., 1995). Note that low-ionic-strength buffer, which contained 0.05M NaCl, 0.05M potassium-phosphate, and 5 mM EDTA at pH 7.0, was used in extraction of sarcoplasmic protein fraction instead of distilled water. Electrophoretic results suggested that myosin and actin were the major proteins in the myofibrillar protein fractions for all species. In contrast, sarcoplasmic protein fractions contained neither myosin nor actin (SDS-PAGE data not shown).

Analysis of variance (Table 2) indicated that species and protein concentration had significant effects on all gel strength characteristics and cooking loss. Protein concentration had significant quadratic effects on all characteristics except Ea. Significant interactions between species and protein concentration on all gelation properties were observed.

The dependence of gel strength and cooking loss on protein concentrations was determined at pH 6.0 because most muscles had higher gel strength at pH 6.0 in our previous study (Lan et al., 1995). Myofibril gels were prepared using 5, 7, 8, 9, and 10% protein for CB, CT, TB, and TT, and 5, 7, and 10% protein for pork, beef and fish myofibrils. Force required to move plunger through myofibril gels (Fig. 1), force required to rupture gels (Fig. 2), viscosity index of gels (Fig. 3), and apparent elasticity values (Fig. 4) increased, and cooking losses (Fig. 5) decreased with increasing protein concentrations. Differences in gel strengths (Fp, Pf, and Ni in Fig. 1, 2, and 3) among species were very small at 5% protein; however, as protein concentration increased, the magnitude of the difference increased between fish and most other species. Fish myofibrils had the lowest Fp and Ni and the highest cooking loss compared to most other species at 7% (Table 4) and 10% (Table 5) proteins. TT myofibrils had lower Fp and Ni at 5% (Table 3) and 7% protein, and
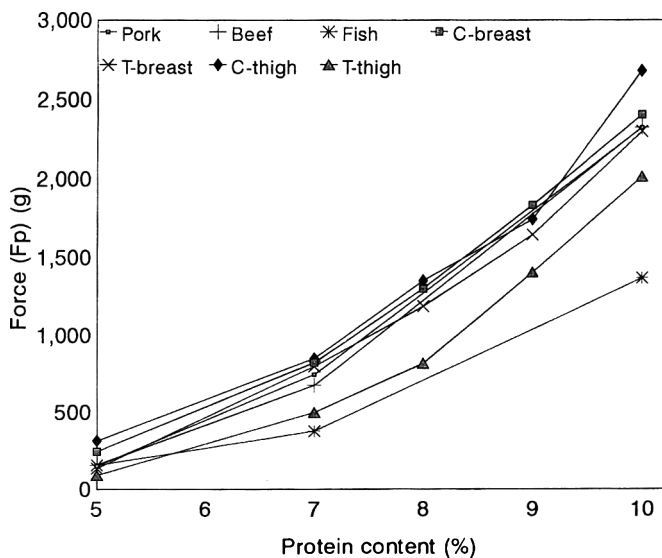
Fig. 1—Effect of protein concentration on force required to move plunger through myofibril gel (Fp) of different species. Means of eight replicates, C = chicken; T = turkey. Gelation conditions: 2% NaCl, 0.4M MES at pH 6.0; heating from 30 to 70°C at 0.7°C/min. (SE bars are excluded from Fig. 1-5 for visual clarity).

Fig. 2—Effect of protein concentration on force required to rupture myofibril gel (Pf) of different species. Means of eight replicates, C = chicken; T = turkey. Gelation conditions: 2% NaCl, 0.4M MES at pH 6.0; heating from 30 to 70°C at 0.7°C/min.

Fig. 3—Effect of protein concentration on viscosity index (Ni) of myofibril gel of different species. Means of eight replicates, C = chicken; T = turkey. Gelation conditions: 2% NaCl, 0.4M MES at pH 6.0; heating from 30 to 70°C at 0.7°C/min.
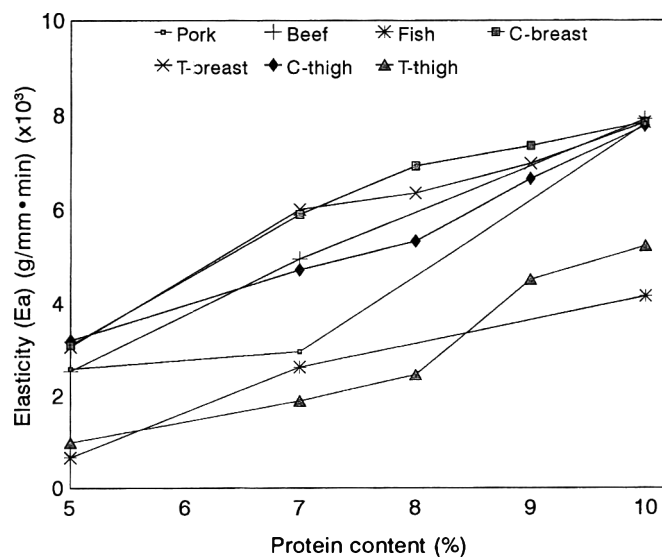
Fig. 4—Effect of protein concentration on elasticity (Ea) of myofibril gel of different species. Means of eight replicates, C = chicken; T = turkey. Gelation conditions: 2% NaCl, 0.4M MES at pH 6.0; heating from 30 to 70°C at 0.7°C/min.

higher cooking loss at 7 and 10% protein than TB myofibrils (P < 0.05). At 10% protein, gel strength (Fp, Pf, and Ni) was similar between TB and TT myofibrils (Table 5). Similar gel characteristics were observed at 7% (Fp and Pf) and 10% (Fp, Pf, Ni, and EA) protein gels between CB and CT. However, CB myofibrils had the lowest cooking loss at 7 and 10% protein gels, consistent with the findings in ground muscle systems (Lan et al., 1995).

Gelation characteristics of pork and beef myofibrils were generally intermediate at various protein levels. Elasticity was lower (P < 0.05) for fish and TT gels at each myofibril protein content, while similar elasticity values were observed in 10% myofibril gels for pork, beef, CT, and TB. Results suggest that differences in gelation properties between species were not consistent at various protein concentrations. Ferry (1948) suggested that, in extremely dilute solutions, protein association tendency was satisfied within a single molecule, resulting in more compact molecular configuration and a decrease in intrinsic viscosity. In more concentrated solutions, association may occur between segments of different molecules with network formation. Using a torsion twisting test, Lavelle and Foegeding (1993) and Northcutt et al. (1993) reported that, at 8% protein, turkey breast and thigh myofibril gels had equivalent rheological properties. They suggested that protein isoforms were not the major factor influencing gel structure formation. However, using low protein concentrations (< 2.5%) and compression tests, Dudziak et al. (1988), Foegeding (1987), and Xiong and Brekke (1990 and 1991) reported that gels from myosin and actomyosin (pH 7.0) or salt-soluble proteins (pH 6.0) from turkey or chicken breast had higher shear stress and strain values than those of similar proteins from thigh. Contradictory results may be related to amounts and types of proteins used. This suggested that interactions among protein molecules at a concentration high enough to form a stronger gel matrix may differ from interactions occurring in dilute protein suspensions. It also suggests that rheological procedures used to measure different physical properties may influence results.
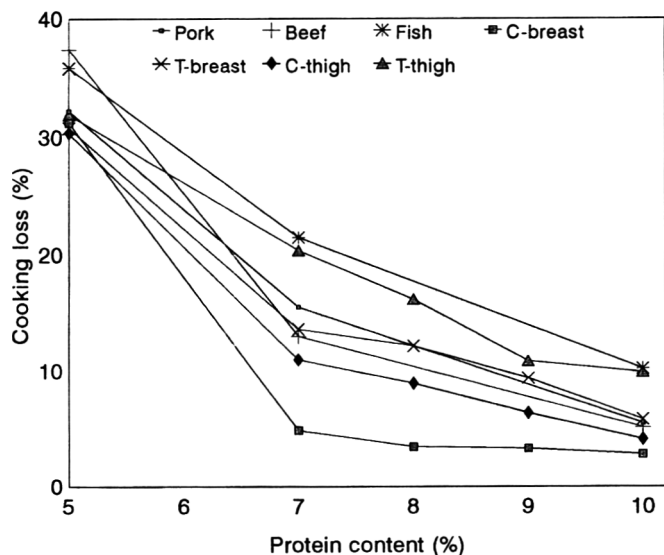
Fig. 5—**Effect of protein concentration on cooking loss of myofibril gel of different species.** Means of eight replicates, C = chicken; T = turkey. Gelation conditions: 2% NaCl, 0.4M MES at pH 6.0; heating from 30 to 70°C at 0.7°C/min.

Fish myofibrils had lower gel strength (Fp, Pf, and Ni) and higher cooking loss than most other species at protein > 5%. This may indicate that fish proteins are more susceptible to denaturation due to freezing than are mammalian or avian muscle proteins. Matsumoto (1978) reported that, when surimi was stored at −20°C without addition of antidenaturants, the protein denatured markedly and, in turn, lost its gel forming ability. However, the cryoprotectant effects of sorbitol, sucrose and polyphosphate could prevent denaturation of frozen surimi. In addition, Cheng et al. (1979) and Deng (1981) reported that the degradation of myosin was associated with an alkaline protease,

resulting in reduction of textural strength of fish gels, especially when cooked at 50–70°C. We could also postulate that protein denaturation in frozen fish muscle reduced extractability of sarcoplasmic protein, resulting its lower percentage in our results. Similar results were obtained by Park (1991) who compared the gel forming abilities of surimi-like materials from beef, pork (with 0.5% polyphosphate and 4% sorbitol added) and commercial surimi by measuring gel hardness after heating from 35 to 80°C at 0.5°C/min. Myofibrils from beef or pork formed gels with greater hardness than commercial fish surimi above 55°C. As concentration of myofibrillar protein in the gel increased from 7 to 15%, hardness of gels increased, regardless of cooking temperature or species. Cofrades et al. (1993) evaluated the effects of protein concentration, pH and ionic strength on viscosity of actomyosin from chicken breast, pork loin and fish (hake). Results suggested that protein concentration and pH increased viscosity of actomyosin gels; however, no inter-species differences were reported. The absence of species differences may be due to the low protein concentration (0.5–1.5%) they used.

At 5% protein, CT myofibrils produced the strongest (P < 0.05) gels (Table 3); at higher protein contents, CT myofibrils maintained a higher gel strength than the other myofibrils (Tables 4 and 5). The collagen content of CT myofibril was highest (0.68 mg/g) and CB was the second highest (0.43 mg/g) among all species (0.11 to 0.19 mg/g for the other 5 myofibrils). It is not clear whether the higher gel strength was related to collagen content. Bailey (1988) suggested that epimysium contained a high proportion of heat-soluble cross-linkages, while the perimysium and endomysium had a higher concentration of relatively heat-stable cross linkages. Reductions in collagen solubility decrease thermal breakdown of collagen. Bailey and Light (1989) also suggested that in meat connective tissues the amount of collagen solubilized by heating was only 10–15%. Denatured collagen is important as an emulsifier, stabilizer, binding agent, and also as a gelling and thickening agent. The collagen content in CT and CB myofibrils may have contributed to the higher gel strength.

Table 3—Effect of species on thermal gelation of myofibrils at 5% protein[g]

Traits	Pork	Beef	Fish	CB	CT	TB	TT	SEM
				Myofibril species				
Fp (g)	105[d]	157[c]	156[c]	245[b]	315[a]	136[c]	91[d]	7.49
Pf (g)	87[de]	97[d]	132[c]	161[b]	225[a]	87[de]	64[e]	9.05
Ni (×10⁻³) (g · min/min²)	1.49[d]	1.64[cd]	1.73[c]	2.63[b]	3.20[a]	1.45[d]	1.00[e]	0.08
Ea (g/mm · min)	2585[a]	2535[ab]	661[c]	3096[a]	3198[a]	3061[a]	984[bc]	596
Cooking loss (%)	33.76[b]	37.41[a]	36.10[a]	31.25[c]	30.30[c]	30.80[c]	31.92[c]	0.64

[a-f] Means in same row with different superscript differ (P < .05).
[g] Where: Fp = Force to move plunger through gel; Pf = Force to rupture gel; Ni = Viscosity index; Ea = Elasticity; SEM = standard error of means.

Table 4—Effect of species on thermal gelation of myofibrils at 7% protein[g]

Traits	Pork	Beef	Fish	CB	CT	TB	TT	SEM
				Myofibril species				
Fp (g)	745[bc]	675[c]	377[e]	822[ab]	850[a]	797[ab]	497[d]	27.96
Pf (g)	465[c]	555[bc]	252[d]	667[ab]	720[a]	640[ab]	317[d]	44.73
Ni (× 10⁻³) (g · min/mm²)	7.36[c]	6.92[c]	3.75[e]	8.61[b]	9.71[a]	7.75[c]	4.80[d]	0.29
Ea (g/mm · min)	2957[bcd]	4952[ab]	2624[dc]	5900[a]	4715[abc]	6005[a]	1888[d]	758
Cooking loss (%)	15.53[b]	13.02[c]	21.49[a]	4.86[e]	10.99[d]	13.62[bc]	20.38[a]	0.69

[a-f] Means in same row with different superscript differ (P < 0.05).
[g] Where: Fp = Force to move plunger through gel; Pf = Force to rupture gel; Ni = Viscosity index; Ea = Elasticity; SEM = standard error of means.

Table 5—Effect of species on thermal gelation of myofibrils at 10% protein[g]

Traits	Pork	Beef	Fish	CB	CT	TB	TT	SEM
				Myofibril species				
Fp (g)	2320[abc]	2322[abc]	1366[e]	2401[ab]	2680[a]	2296[bc]	2010[c]	120
Pf (g)	1750[cd]	2250[ab]	1672[d]	2357[a]	2410[a]	2290[ab]	2040[bc]	90.57
Ni (× 10⁻³) (g · min/mm²)	21.71[a]	22.56[a]	13.33[b]	20.09[a]	23.01[a]	19.90[a]	22.09[a]	1.17
Ea (g/mm · min)	9397[ab]	10562[a]	4152[d]	7850[b]	7772[b]	7842[b]	5230[cd]	752
Cooking loss (%)	5.49[bc]	5.13[bc]	10.24[a]	2.80[d]	4.10[c]	5.71[b]	9.92[a]	0.44

[a-f] Means in same row with different superscript differ (P < 0.05).
[g] Where: Fp = Force to move plunger through gel; Pf = Force to rupture gel; Ni = Viscosity index; Ea = Elasticity; SEM = Standard error of means.

CONCLUSIONS

DIFFERENCES EXISTED in gelation properties of myofibrils between species and muscle types. Myofibrils from chicken breast and thigh had the highest gel strength and water holding capacity, while fish myofibrils had the lowest. Pork and beef myofibrils were intermediate and gelation properties of different types of myofibrils were altered by varying protein concentrations. This suggested that interactions among protein molecules in a high concentration gel matrix may differ from those occurring in dilute protein suspensions.

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Ms received 10/14/94; revised 3/16/95; accepted 4/8/95.

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that increasing the pH above 6.0 in processed meat would improve protein binding and water binding.

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Ms received 10/14/94; revised 3/25/95; accepted 5/16/95.

Nitrite Effects on Formation of Volatile Oxidation Products from Triolein

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ABSTRACT

Oxidation of triolein resulted in the formation of 5 major aldehydes (heptanal, octanal, nonanal, 2-decenal and 2-undecenal). Pre-treatment of the triolein with a twofold molar excess of N_2O_3 did not qualitatively affect the compounds formed but reduced the amount formed by 63 to 74%. Treatment with tenfold excess of N_2O_3 resulted in formation of several unknown compounds. However, the ratio of N_2O_3 to olefinic groups in lipids in cured meats was less than 1:750. These data indicate that nitrite reduced the formation of aldehydes from lipid oxidation and contributed to anti-oxidant activity. However, the amount of nitrite added to meats is probably insufficient for this to be a major anti-oxidant mechanism.

Key Words: nitrite, lipid oxidation, cured meats, triolean. fats

INTRODUCTION

THERE ARE EXTENSIVE published reports on the effects of nitrite (NO_2^-) salts in cured meat (National Research Council, 1981; Hotchkiss and Cassens, 1987). Nitrite imparts some desirable characteristics including color fixation (Cassens et al., 1979), anti-microbial activity (Sofos et al., 1979), and inhibition of lipid oxidation (Pearson et al., 1977). The anti-oxidative properties of NO_2^- are important because the flavor characteristics of cured meats probably derive primarily from inhibition of lipid oxidation rather than formation of specific cured meat flavors (Gray et al., 1981).

The anti-oxidant activity of NO_2^- cannot be explained by its direct anti-oxidative potential. Evidence supports at least 3 indirect mechanisms which may operate simultaneously (Igene et al., 1985). Nitrite may act by: binding heme and preventing release of catalytic iron; binding non-heme iron thus inhibiting catalysis; and/or stabilizing olefinic lipids against oxidation. Stabilization of the porphyrin ring, preventing release of Fe^{+2} during cooking, was considered to be the most important mechanism by Igene et al. (1985). This mechanism was also supported by Kanner et al. (1980) who compared the effects of nitric oxide myoglobin (MbNO), metmyoglobin (Mmb) and oxymyoglobin (MbO_2) on lipid oxidation in model systems. While Mmb and MbO_2 had a clear pro-oxidative effect, MbNO under the same conditions acted as an anti-oxidant. Anti-oxidative activity was maintained in the presence of pro-oxidants such as heme proteins. The anti-oxidant activity of iron-nitric oxide complexes has also been studied (Kanner et al., 1984). Cysteine in the presence of iron ions caused rapid oxidation but the cysteine-Fe-NO complex had no pro-oxidant effect.

Less evidence concerns the effects of NO_2^- binding to unsaturated fatty acids. Goutefongea et al. (1977) first demonstrated NO_2^- binding to fatty acids and triglycerides which was related to the degree of unsaturation. Walters et al. (1979) reported reaction of palmitodiolein with oxides of nitrogen. Zubillaga et al. (1984) extracted polar lipids from raw and NO_2^--treated beef and pork and found that NO_2^--treated lipids had substantial activity inhibiting oxidation of linoleic acid. They suggested neither residual NO_2^-, carbon-nitroso or nitrogen-nitroso com-

pounds, nor products of the addition of nitrogen oxides to olefins, accounted for the anti-oxidant activity. Ross et al. (1987) demonstrated that dinitrogen trioxide (N_2O_3) reacted with methyloleate to form several addition products, some of which had N-nitrosation capacity under conditions similar to frying bacon. Dinitrogen trioxide is the reactive species formed when NO_2^- is added to mildly acidic media such as cured meats (Challis and Kyrtopoulos, 1978). Later, Liu et al. (1988) identified covalent nitro-nitroso derivatives of olefins which decomposed to nitrosating agents upon heating to frying temperatures. These data showed that N_2O_3 added to olefinic fatty acids and suggested that N_2O_3 protected olefinic fatty acids against oxidation. This hypothesis was supported by Freybler et al. (1993) who extensively studied the ability of NO_2^- to stabilize lipids against peroxidation. Using lipids extracted from pork samples, they demonstrated that NO_2^- stabilized unsaturated lipid fractions against oxidation based on significant differences in 2-thiobarbituric acid (TBA) values for lipid fractions isolated from NO_2^--treated and non-treated pork samples. They concluded that NO_2^- exerted at least part of its anti-oxidant activity by binding and thus stabilizing unsaturated membrane lipids.

These data indicated that NO_2^- to some extent inhibited lipid oxidation by binding to olefinic lipids. No report has explored the extent to which NO_2^- stabilizes lipids against oxidation by quantification of volatiles produced by oxidation. Our objective was to determine the effects of N_2O_3 treatment on the formation of volatile products from olefinic lipid oxidation. Formation of such volatiles leads to objectionable odors resulting from meat oxidation (Ajuyah et al., 1993).

MATERIALS & METHODS

Reagents

Triolein (1,2,3-tri[cis-9-octadecenoyl]glycerol) was purchased from Sigma Chemical Co. (St. Louis, MO). Dinitrogen trioxide (N_2O_3) was synthesized by the method of Ross et al. (1987). A two-stage enclosed glass micro-trap was purged with N_2 and perchloric acid (5 mL, 70%) slowly added to sufficient solid sodium nitrite to form $\approx$ a twofold excess of N_2O_3 to double bonds in 500 mg of triolein. The N_2O_3 was purged from the acid-nitrite mixture with N_2 and frozen as a characteristic light blue solid in a trap cooled by liquid nitrogen (LN_2). Purified O_2 and N_2 were acquired from Airco Products (Allentown, PA). Hexane was analytical reagent grade.

Apparatus

An all glass thermal oxidation/collection apparatus was used to continuously purge and trap volatiles produced from triolein oxidation (Fig. 1). Triolein was oxidized in an atmosphere of prepurified O_2 without catalysts or solvent in a 50-mL glass impinger (Wheaton, Millville, NJ) at 90°C. A continuous O_2 flow of 40 mL/min was maintained through the tube in order to purge oxidation products as they were formed. The tube was connected to two glass vapor traps (50-mL Wheaton impingers) with glass tubing fitted with stopcocks and surrounded by heating tape to prevent condensation. The glass traps were immersed in LN_2. Each trap was linked to a water trap to compensate for the vacuum created by the low temperature and to prevent contaminants from entering the traps. The traps were efficient enough to condense all O_2 flowing through the system. Lack of background interference was established by conducting experiments with all reagents (including triolein) but with N_2 replacing the O_2.
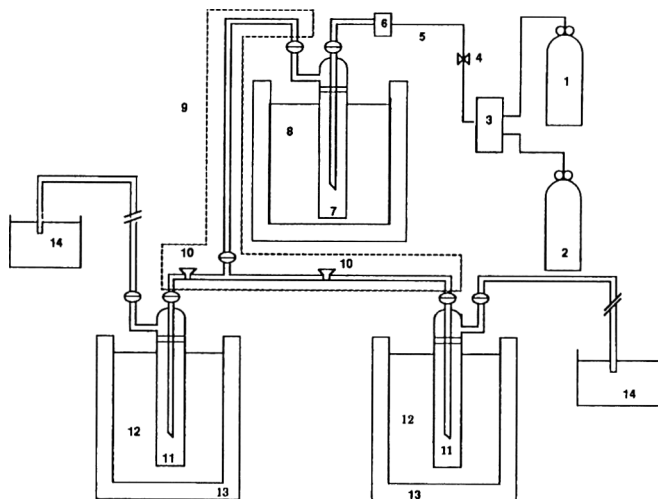
Fig. 1—Apparatus to react triolein with O₂ and collect volatiles. (1) compressed O₂; (2) compressed N₂; (3) gas blender; (4) valve; (5) metal tubing; (6) metal-to-glass joint; (7) reaction tube; (8) water bath; (9) heated zones; (10) stopcocks; (11) glass impinger traps; (12) LN₂; (13) Dewar flasks; (14) water trap.

Reaction with N_2O_3

Triolein (500 mg; 0.56 mmol) in 3 mL hexane was added to the blue solid N_2O_3 and the mixture held at $-77°C$ in a dry ice/methoxyethanol bath for 12 hr. The sample was vacuum-evaporated to remove unreacted N_2O_3 and hexane, and the resulting N_2O_3-treated triolein was reacted with O_2.

Oxidation procedure

Triolein (500 mg; 0.56 mmol), or the N_2O_3-treated triolein, was placed in the reaction tube, the system sealed, and the tube put in a 90°C water bath. Oxygen (40 mL/min) was passed through the tube and into the LN₂-cooled traps. At predetermined time intervals, flow from the oxidation tube was switched from one trap to the other. Volatiles were then extracted from the trap with 4 mL of ultra-pure Freon-113 (1,1,2-trichloro-1,2,2,-trifluoroethane). Freon extracts were stored at $-5°C$ in 10-mL glass vials equipped with aluminum foil liner caps (Krackeler Scientific, Inc., Albany, NY) until analysis. For subsequent analysis, 1 mL of Freon extract was concentrated to 0.2-mL in a slow N₂ gas stream.

Analysis of volatiles

A Hewlett Packard 5790A gas chromatograph, equipped with a 0.53 mm × 30m fused silica capillary column coated with Rtx-50 (50% phenyl-50% methyl polysiloxane; Restek Corp., Bellefonte, PA) was used. Temperatures were as follows: injector, 200°C; detector, 250°C; column, 3 min at 35°C, increased to 225°C at 4°C/min, held at 225°C for 10 min. Carrier gas was N₂ at 2.6 mL/min. Retention times and peak areas were recorded by electronic integrator. Compounds were identified by co-injection with authentic standards. Statistical significance in nonanal formation between N₂O₃-treated and untreated triolein were determined by independent t-test (Mini-tab Data Analysis Software 1.1, 1988; n = 3)

RESULTS & DISCUSSION

ANALYSES OF CONDENSATE, after triolein was heated and purged with N₂, showed minor peaks indicating that the triolein did not contain large amounts of volatile compounds and a lack of interference from the system or reagents. Likewise, purging the system with O₂ when no triolein was present resulted in chromatograms with only minor peaks, which indicated a lack of artifacts.

A typical chromatogram of the volatiles produced from reacting triolein with O₂ at 90°C for 24 hr (Fig. 2a) showed five major peaks identified as heptanal, octanal, nonanal, 2-decenal and 2-undecenal. These were based on co-injection with standards and comparison of retention times. These compounds were identical to those identified by Selke et al. (1977) from triolein oxidation at 196°C for 10 min. In addition to those compounds, Selke et al. (1977) identified heptane and octane as major products. These were likely present in our samples but were eluted with the solvent front and, hence, not recorded peaks. While the above compounds were detected by Selke et al. (1977) in a system containing only triolein, Ajuyah et al. (1993) have recently shown that several of these compounds also occurred in significant amounts in oxidized meats.

Treating triolein with twofold molar excess of N_2O_3/mole of double bonds prior to reacting with O₂ did not qualitatively affect results but substantially reduced the amount of all products formed (Fig. 2b). The amounts were lowered by: 63%, heptanal; 69%, octanal; 68%, nonanal; 74%, 2-decenal; 67%, 2-undecenal; (n = 3). Furthermore, several minor oxidation products present as a result of triolein oxidation were not detected after
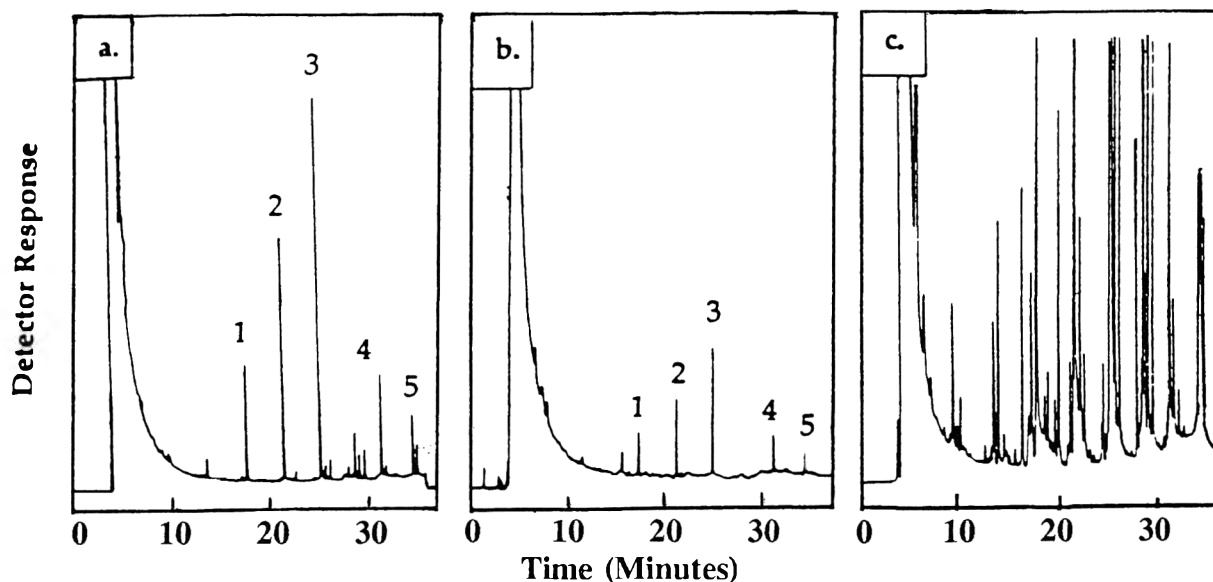
Fig. 2—GC-chromatograms of: (a) volatiles formed when triolein was reacted with O₂ at 90°C for 24 hr; (b) volatiles formed when triolein that had first been treated with a twofold excess of N₂O₃ was further reacted with O₂ at 90°C for 24 hr; (c) volatiles formed when triolein that had been first treated with a tenfold excess of N₂O₃ was further reacted with O₂ at 90°C for 24 hr. Peaks 1 to 5 identified as heptanal, octanal, nonanal, 2-decenal, and 2-undecenal, respectively.

reacting N_2O_3-treated triolein with O_2 under the same conditions (Fig. 2a, 2b).

In agreement with Selke et al. (1977), nonanal was the predominant oxidation product of triolein. The quantities of nonanal formed over time from the reaction of triolein with O_2 at 90°C with and without N_2O_3 treatment were summarized (Fig. 3, n = 3 for each treatment). Several hours were required before sufficient volatiles were collected to be detected. This was probably due in large part to the mild conditions (90°C; absence of catalyst) under which oxidation occurred. The difference in amounts of nonanal formed at 24 hr were highly significant (p ≤ 0.01) between the untreated and N_2O_3-treated triolein.

The data indicated that N_2O_3 substantially inhibited, but did not completely block the formation of volatile oxidation products. The degree to which N_2O_3 treatment protects the double bonds from oxidation would depend, in part, on the number of double bonds bound by N_2O_3. The mass (n = 3) of a 500-mg triolein sample increased by 108 mg after reacting with N_2O_3. This corresponds to 1.42 mmole of N_2O_3 and suggests that only 85% of the double bonds in the triolein had reacted with N_2O_3. Presumably, double bonds which had not reacted with N_2O_3 would be available to react with O_2. Thus, inhibition of oxidation was not expected to be complete.

Increasing the ratio of N_2O_3 to double bonds from a two- to a tenfold molar excess resulted in a complex chromatogram after the products were reacted with O_2 at 90°C (Fig. 2c). Several new compounds were observed indicating that a 10-fold excess of N_2O_3 resulted in formation of compounds not seen at a twofold excess. Some N_2O_3 might have dissociated from triolein as a result of heating employed during oxidation reactions leaving some double bonds available for oxidation. Liu et al. (1988) found that nitro-nitroso compounds derived from olefins decomposed at ≈ 100°C.

Our studies used a molar ratio of N_2O_3 to olefinic groups of $\approx 2:1$. However, in cured meats, olefinic groups are in large excess compared to NO_2^-. For example, the addition of 120 mg/kg sodium nitrite to a pork product is equivalent to 0.87 mmol of N_2O_3/kg of meat ($2NaNO_2 + 2H^+ \rightarrow N_2O_3 + H_2O + 2Na^+$). Assuming a pork product contains 30% fat of which 42% is oleic and 9.6% linoleic acid (Bogert et al., 1973) the concentration of olefinic groups would be 0.66M. Thus the ratio of N_2O_3 to olefinic groups is $<1:750$. While N_2O_3 was effective at inhibiting oxidation by stabilizing olefinic groups with which it had reacted, the amount of NO_2^- added to meats was insufficient to be a considerable mechanism for inhibiting oxidation (in our opinion). However, oxidation may occur primarily in membrane lipids which are highly unsaturated and a small amount of nitrite might be more effective than our calculations imply.

Formation of the major volatile products of lipid oxidation was inhibited by NO_2^- to olefinic lipids. This provided further evidence that NO_2^- could inhibit the formation of compounds contributing to oxidized flavor (i.e., warmed-over flavor) in meats. Our results were consistent with observations of Freybler et al. (1993) based on TBA values. They concluded that NO_2^- (or its reactive product, N_2O_3) inhibited oxidation by stabilizing double bonds. A mechanism for reduction in aldehyde formation and lowering of TBA values is consistent with the formation of C-nitro-nitroso addition to olefinic groups identified by Liu et al. (1988). Our data did not support the conclusions of Zubillaga et al. (1984) that the anti-oxidant activity of sodium nitrite, although associated with the polar-lipid fraction in raw beef or pork but did not appear to involve addition of nitrogen oxides to olefinic double bonds.

Our data were developed in a simple system compared to cured meats and do not directly prove that a similar mechanism applies to meats, nor do they give the relative importance of different anti-oxidant mechanisms of NO_2^-. However, the pres-
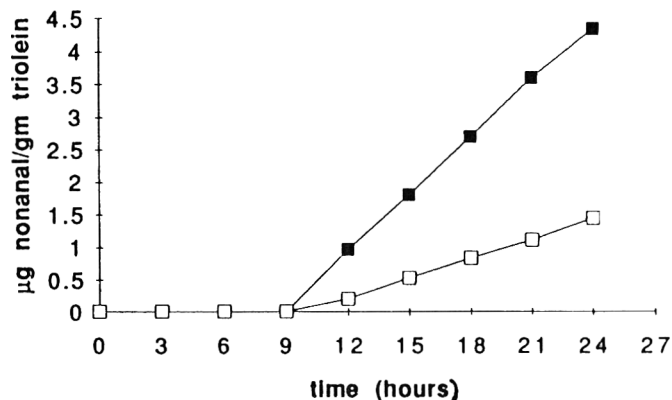
Fig. 3—Formation of nonanal over time when triolein was reacted with O_2 at 90°C for 24 hr (■) or when triolein that had first been treated with a twofold excess of N_2O_3 was further reacted with O_2 at 90°C for 24 hr (□).

ence of lipid-bound NO_2^- in meats has been conclusively demonstrated and confirmed (Hotchkiss et al., 1985; Freybler et al., 1993) as well as the inhibitory effects of such binding on oxidation. The numbers of olefinic groups stabilized compared to those available for oxidation need to be determined for the relative importance of this mechanism to be quantified.

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Ms received 3/13/95; revised 4/12/95; accepted 4/21/95.

Protein Recovery from Veal Bones by Enzymatic Hydrolysis

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——————— ABSTRACT ———————

Enzymatic hydrolysis (commercial enzyme) was applied to crushed bones in a batch reactor. The extraction was modeled using a Doehlert design. The effects of temperature (five levels ranging from 40 to 60°C), pH (seven levels from 5.7 to 7.5), enzyme concentration (seven levels from 20 to 80 g.kg^{-1} protein), hydrolysis time (seven levels from 30 to 180 mn) and protein concentration (three levels from 60 to 90 g.kg^{-1} mixture) were studied. Response surface methodology (RSM) predicted changes in protein yield and hydrolysis degree. Duration and enzyme concentration were the most important parameters, whereas pH and temperature exhibited no significant effects. Inhibition was observed over 80 g.kg^{-1} of protein concentration.

Key Words: veal, protein, enzymatic hydrolysis, meat by-product, Doehlert-matrix

INTRODUCTION

BONE is one of the most plentiful by-products in meat processing varying between 8% and 12% of live weight, depending on species. A high percentage of this potentially valuable protein source is discarded or used as feed and fertilizer (Duerr and Earle, 1973; Jobling, 1986). Nonconventional protein potentials such as meat bone protein should be utilized through specific processes. Several such studies have been reported. Meat proteins from alkaline extracts were recovered from meat offal (Ledward and Lawrie, 1984) and beef bones (Jelen et al., 1979; Dondero et al., 1987). However, the alkali treatment of proteins causes reactions which may be undesirable for food use, such as racemization of amino acid residues, and elimination and addition reactions which may lead to toxic effects (Shih, 1992). Enzymatic treatment has several advantages over chemical modifications minimizing nutrient loss (Adler-Nissen, 1986). By using hydrolysis, Fik and Surowka (1987) prepared a protein concentrate from broiler chicken heads. Sorensen and Rasmussen (1989) developed an enzyme application for upgrading mechanically recovered meat.

Enzymatic hydrolysis of proteins is a complex process because of several peptide bonds and their specific accessibility to enzymatic reactions. This usually depends on temperature, pH, agitation, protease specificity, concentration of enzyme and protein. Adler-Nissen (1982) investigated the influence of four factors (degree of hydrolysis or DH, temperature, enzyme/protein ratio, and the nature of the substrate) on functional properties of soy hydrolysates. Using an incomplete two- and three-level matrix block (DH, substrate, and temperature), they only accounted for the first-order interactions.

Our objective was to determine the relative importance of pH, temperature, time, and enzyme and protein concentrations on responses (DH and yield), because the main obstacle for hydrolysate use in food preparation was bitterness. Due to economic constraints or toxicological considerations, commercial methods for removal of bitter peptides are not available. Therefore, preventing or minimizing formation of bitter peptides can be

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achieved by strict control of proteolytic reactions. We developed an empirical model to account for the overall interactions between parameters and allow selection of the most efficient monitoring parameters on a larger scale. We used response surface methodology (RSM) according to the Doehlert uniform shell design for five factors (Doehlert, 1970).

MATERIALS & METHODS

Preparation, composition and storage of substrate

Fresh food grade veal bones, from the production line of a local salting plant, were used. After crushing twice with a 4-mm-diameter meat grinder, at 4°C, the material was vacuum-packed in 400-g polyethylene bags and stored at −30°C. Samples were subsequently taken from this raw material. The crushed bones contained, per kg: 31.5 g (σ=4.1) total nitrogen, 114.1g (σ=5) fat, 110.7g (σ=5.9) ash and 424g (σ=4.1) dry matter (total solids). Analytical results were obtained from 6 independent assays and the dispersion was expressed by standard error of mean.

Enzyme and reactor experiments

Neutrase® 0.5L is a liquid food grade preparation of *Bacillus subtilis*. The residual proteolytic activity was obtained according to the N,N-dimethyl casein gel diffusion method (Novo Industri, 1979). All assays were batch experiments, in which 300g of crushed/ground bones were suspended in distilled water in a stirred and thermostated, jacketed vessel. The mixture was subjected to the action of protease according to the experimental plan (Table 1). The protein extraction procedure was adapted from the process of Sorensen and Rasmussen (1989). Most of the food grade fat fraction was readily skimmed off the aqueous mixture after heat treatment (Moss and Trautman, 1972). The batch was cooled to the proper temperature, the pH adjusted and a slightly diluted enzymatic solution added. After a given time, the enzyme was inactivated by holding the temperature at 90°C for 15 min with live steam injection. The protein hydrolysate was recovered by screening (30-mesh) and centrifuged (15 min, 2°C, 1000 × g) to remove the residual fat layer. Supernatant and sludge were freeze-dried and stored at −30°C; insoluble residues were dried and weighed.

Chemical composition

The crushed bones and hydrolysates were analyzed for nitrogen content using the Kjeldahl method and fat content was determined by Soxhlet extraction, using standardized analytical methods (AOAC, 1984). Dry matter was determined at 105°C. Ash was obtained by mineralization at 520°C over 24 hr. Amino acid determinations were carried out on acid hydrolysates using a 7300 Beckman amino acid analyzer, according to the procedure of Simpson et al. (1976). Samples were freeze-dried in a LYOVAC freeze-drier Model GT3 (Leybold-Heraeus, Orsay, France) at room temperature (22–25°C).

Table 1—Level distribution of the process variables (bold values: center of the experimental domain)

Process variable	Experimental value	Step	Level
X1: temperature (°C)	40 45 **50** 55 60	10.000	5
X2: pH (pH units)	5.7 6.0 6.3 **6.6** 6.9 7.2 7.5	1.039	7
X3: enzyme concentration (g kg^{-1} protein)	20 28 43 **50** 57 72 80	36.764	7
X4: hydrolysis time (mn)	30 45 90 **105** 120 165 180	94.816	7
X5: protein concentration (g kg^{-1} mixture)	60 **75** 90	19.354	3

Table 2—Doehlert plan and experimental responses

Exp no.	Process variables					Response variables		
	X1	X2	X3	X4	X5	Y1	Y2	Y3
1	1.000	0.000	0.000	0.000	0.000	50.89	65.61	4.95
2	-1.000	0.000	0.000	0.000	0.000	49.45	65.28	4.72
3	0.500	0.866	0.000	0.000	0.000	41.15	63.11	4.32
4	-0.500	-0.866	0.000	0.000	0.000	48.18	67.09	4.98
5	0.500	-0.866	0.000	0.000	0.000	48.74	68.86	4.92
6	-0.500	0.866	0.000	0.000	0.000	50.83	66.11	4.78
31a	0.000	0.000	0.000	0.000	0.000	47.18	67.34	4.91
7	0.500	0.289	0.816	0.000	0.000	53.24	69.99	6.47
8	-0.500	-0.289	-0.816	0.000	0.000	47.11	59.81	3.85
9	0.500	-0.289	-0.816	0.000	0.000	52.47	63.11	6.20
10	0.000	0.577	-0.816	0.000	0.000	46.85	62.47	4.19
11	-0.500	0.289	0.816	0.000	0.000	53.75	69.68	6.80
12	0.000	-0.577	0.816	0.000	0.000	52.47	69.61	6.10
31b	0.000	0.000	0.000	0.000	0.000	47.93	67.45	4.92
13	0.500	0.289	0.204	0.791	0.000	55.69	68.62	6.38
14	-0.500	-0.289	-0.204	-0.791	0.000	47.65	59.48	4.90
15	0.500	-0.289	-0.204	-0.791	0.000	49.23	67.06	5.10
16	0.000	0.577	-0.204	-0.791	0.000	46.07	62.61	4.01
17	0.000	0.000	0.612	-0.791	0.000	42.27	61.99	3.13
18	-0.500	0.289	0.204	0.791	0.000	50.80	69.88	4.74
19	0.000	-0.577	0.204	0.791	0.000	52.27	72.09	6.17
20	0.000	0.000	-0.612	0.791	0.000	49.83	68.43	4.70
31c	0.000	0.000	0.000	0.000	0.000	45.81	66.78	4.80
21	0.500	0.289	0.204	0.158	0.775	53.60	59.92	6.51
22	-0.500	-0.289	-0.204	-0.158	-0.775	53.15	69.07	6.95
23	0.500	-0.289	-0.204	-0.158	-0.775	54.12	71.86	6.80
24	0.000	0.577	-0.204	-0.158	-0.775	45.72	69.38	6.90
25	0.000	0.000	0.612	-0.158	-0.775	54.91	72.39	7.90
26	0.000	0.000	0.000	0.632	-0.775	54.91	71.72	8.05
27	-0.500	0.289	0.204	0.158	0.775	51.60	63.58	5.70
28	0.000	-0.577	0.204	0.158	0.775	51.52	68.88	5.15
29	0.000	0.000	-0.612	0.158	0.775	44.20	59.98	3.67
30	0.000	0.000	0.000	-0.632	0.775	44.14	58.01	3.44
31	0.000	0.000	0.000	0.000	0.000	47.10	66.37	4.85

Fig. 1—Effect of time on the extent of hydrolysis degree (DH) (○) and on the corrected osmolality (mOsm) (△) of crushed bone dispersion by Neutrase 0.5L at the center point. Reaction conditions: 50°C; pH 6.6; enzyme, 50g kg^{-1} meat protein; protein concentration in the reactor, 75g kg^{-1} mixture.

Table 3—Analysis of variance for hydrolysis process data: polynomial model

Source of variation	Sum of square			Degrees of freedom	Mean square		
	Y1	Y2	Y3		Y1	Y2	Y3
Regression	382.27	495.45	44.13	20	19.11	24.77	2.21
Residual	87.96	42.55	5.65	13	6.76	3.27	0.44
Lack of fit	85.64	41.79	5.64	10	8.56	4.18	0.56
Pure error	2.32	0.76	0.009	3	0.77	0.25	0.00
Total	470.23	538.00	49.78	33			
R^2	0.82	0.93	0.89				
Adjusted R^2	0.53	0.80	0.72				

Degree of hydrolysis (DH)

Reaction kinetics were followed by measuring the degree of hydrolysis with the pH-stat method according to the procedure of Adler-Nissen (1986). Above pH 6.5, the dissociation of amino groups becomes significant and the equation relating DH to alkali consumption during the course of the hydrolysis reaction is given by:

$$DH\ (\%) = \frac{1}{\alpha \cdot h_{tot}} \cdot \frac{B \cdot N_B}{MP} \cdot 100$$

where DH is defined as the rate of the number of peptide bonds cleaved (h) over the total number of such bonds in the protein substrate (h_{tot}). This is calculated from the amino acid composition of the substrate (Table 5), and can be approximated to 7.8 equivalent per kg protein (calculated as Kjeldahl N × 5.25); B is the alkali consumption in mL; N_B is the normality of the alkali (4N NaOH); MP is the grams of protein (N × 6.25) in the reactor. The degree of dissociation is

$$\alpha = \frac{10^{pH-pK}}{1 + 10^{pH-pK}}$$

The pK values at different temperatures T were calculated from Steinhardt and Beychok (1964):

$$pK = 7.8 + \frac{(298 - T)}{298 \cdot T} \cdot 2400$$

In the range of pH 5.7–6.5, the osmometer technique was used to monitor the DH according to the procedure of Adler-Nissen (1986). The measurements are not impaired by the presence of insoluble matter in the protein hydrolysis reaction mixture. Correlations between osmolality and conductivity (R^2 = 0.99) were used to correct the osmometer readings which can be affected by addition of alkali to keep constant pH during hydrolysis. The osmometer was calibrated against the pH-stat method leading to direct estimation of DH % when the osmolality of a given sample is known (R^2 = 0.90). The least square regression gave:

$$\% \ DH_{(pH - stat \ method)} = 0.12 \ osmolality \ corrected - 2.55$$

Experimental design and statistics

According to preliminary studies based on a survey of the effective parameters, a Doehlert experimental design was selected, using NEM-ROD® software (Mathieu and Phan-Tan-Luu, 1992). This experimental matrix displays a uniform distribution of the points within the experimental domain.

Processing variables investigated were temperature (X1), pH (X2), enzyme concentration (X3), hydrolysis time (X4) and protein concentration in the reactor (X5). The Doehlert matrix allowed a number of distinct levels for each variable. The first was a five-level variable, the last one was a three-level and all the others had seven levels (Table 1). According to the experimental constraints, these variables were affected by the Doehlert matrix with respect to the range and the power of each variable, as determined in a preliminary study and published data (Webster et al., 1982; Adler-Nissen, 1986; Quaglia and Orban, 1987; Chambers and Rasmussen, 1988). Responses were:

- hydrolysis yield (Y1) expressed as amount of nitrogen in the hydrolysate over initial nitrogen content.
- the supernatant total nitrogen/hydrolysate total nitrogen (Y2) allowing determination of soluble and insoluble protein fractions in the hydrolysates, respectively.
- the DH (Y3) which should be controlled to avoid product bitterness.

The total number of points (N = k^2 + k + 1) for five factors is 31. Thirty-four experiments were carried out: Experiment 31 was performed at the center of the experimental domain and repeated three times (31a, 31b, 31c) in order to estimate residual variance (Table 2).

A full quadratic model containing 21 coefficients including interaction terms was assumed to describe relationships between responses and experimental factors:

$$\eta_k = f_k (X_1, X_2, X_3, X_4, X_5) \quad \text{where } k = 1, 2, 3 \text{ for } f_k \qquad [1]$$

$$\eta_k = \beta_{k0} + \sum_{i=1}^{5} \beta_{ki} X_i + \sum_{i=1}^{5} \beta_{kii} X_i^2 + \sum_{i=1}^{4} \sum_{j=i+1}^{5} \beta_{kij} X_i X_j \qquad [2]$$

where η_k is the dependent variable; β_0 is the constant coefficient; X_i are

Fixed variables

X2	=	0.000
X4	=	0.000
X5	=	0.000

Response Y1

1	:	41.2
2	:	42.5
3	:	43.8
4	:	45.1
5	:	46.4
6	:	47.8
7	:	49.1
8	:	50.4
9	:	51.7
10	:	53.0
11	:	54.4
R	=	1.000
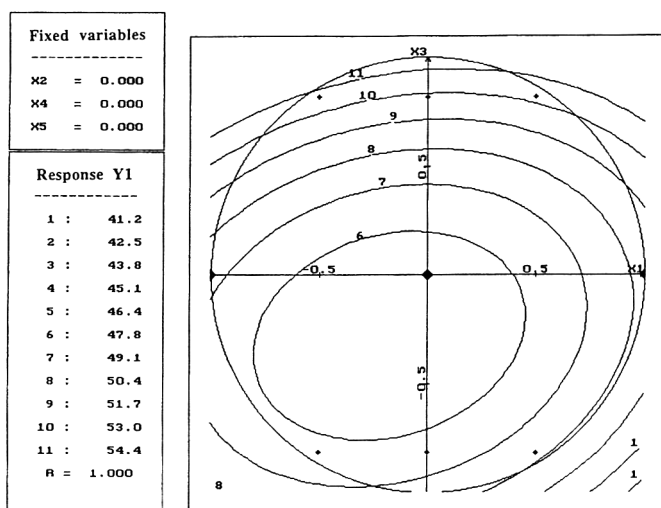
Fig. 2—Response surface contours for protein yield (Y1) as a function of enzyme concentration (X3) and temperature (X1). pH (X2), time (X4) and protein concentration (X5) were kept at 0 coded levels.

Fixed variables

X1	=	0.000
X2	=	0.000
X5	=	0.000

Response Y3

1	:	3.13
2	:	3.58
3	:	4.02
4	:	4.47
5	:	4.92
6	:	5.37
7	:	5.81
8	:	6.26
9	:	6.71
10	:	7.16
11	:	7.60
R	=	1.000
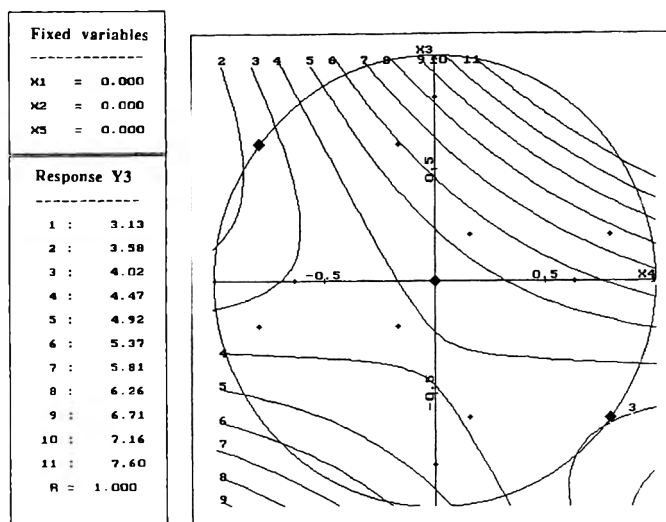
Fig. 4—Response surface contours for the hydrolysis degree (Y3) as a function of time (X4) and enzyme concentration (X3). Temperature (X1), pH (X2) and protein concentration (X5) were kept at 0 coded levels.

empirical model may be useful, particularly if responses, need to be only approximated over a range of variables (Box et al., 1978; Box and Draper, 1987).

Data of the different responses Y1, Y2, Y3 obtained by using the experimental Doehlert matrix led to 92.7% efficiency (Table 2). From this plan, the estimated model coefficients β of Eq. (2) were calculated by regression analysis on experimental responses (REGSIA procedure of the NEMROD® program). Yields and hydrolysis degree varied with significant effect ($p \leq 0.05$) due to hydrolysis time ($\beta4$) and enzyme concentration ($\beta3$) (Y2: $p \leq 0.01$ and Y3: $p \leq 0.05$). An interaction between hydrolysis time and enzyme concentration (model coefficient $\beta34$, $p \leq 0.10$) was observed. No significant trends were found in the pH range ($\beta2$) or temperature ($\beta1$).

ANOVA and canonical analysis were used to characterize the validity of proposed polynomial models. The adequacy of each polynomial model was tested by the lack of fit, the coefficient of determination, R^2, and adjusted R^2. Statistical analysis of responses (Table 3) were accurately predicted by the quadratic model, as shown by coefficients of determination ($R^2 \geq 80\%$).

Fixed variables

X1	=	0.000
X4	=	0.000
X5	=	0.000

Response Y1

1	:	41.2
2	:	42.5
3	:	43.8
4	:	45.1
5	:	46.4
6	:	47.8
7	:	49.1
8	:	50.4
9	:	51.7
10	:	53.0
11	:	54.4
R	=	1.000

Fig. 3—Response surface contours for protein yield (Y1) as a function of pH (X2) and enzyme concentration (X3). Temperature (X1), time (X4) and protein concentration (X5) were kept at 0 coded levels.

the coded independent variables; β_i are the linear coefficients, β_{ij} are the second-order interaction coefficients and β_{ii} are the quadratic coefficients.

Data analysis

Data were computed using the NEMROD® software (Mathieu and Phan-Tan-Luu, 1992) including ANOVA and canonical analysis in order to obtain interaction data between the process variables and responses.

RESULTS & DISCUSSION

Hydrolysis curves

Data were obtained from the time-course experiments using the pH-stat method and osmolality plot for the DH values (Fig. 1). The high initial reaction rate (slope of hydrolysis curve) decreased with time, and the shape of the hydrolysis curve was typical of a substrate-enzyme system (Quaglia and Orban, 1987). The mechanism underlying such a complex process is too complicated to devise a theoretical model. In such case, an

Geometric representation

A series of response surfaces was generated by plotting yields and hydrolysis degree vs two processing variables (the three others were kept at the center of the experimental field). The relationship between hydrolysis yield and two quantitative variables (enzyme concentration and temperature) could be represented by contour surfaces (Fig. 2). In the pH range investigated, changes in yield and amount of different fractions were not significant on the contour plot (Fig. 3). The pH value may not be controlled during hydrolysis, thus simplifying the regulation of the batch reactor, as mentioned by Sorensen and Rasmussen (1989) and Surowka and Fik (1992).

From analysis of the isoresponse contours, enzyme amount and time are apparent prevailing factors affecting resulting DH which ranged from 3 to 8% (Fig. 4), and therefore should be increased in order to improve protein recovery. The isoresponse surfaces of the response Y1 (yield) and the response Y2 (supernatant) followed similar trends. However, the DH (Y3) must be kept at a value lower than 6–8 to avoid an intense bitter flavor which could impair use of protein hydrolysates in human foods (Adler-Nissen, 1986). Protein concentration (X5) seems to have an inhibiting effect on response Y2 (Fig. 5) for a protein concentration >8%. This may be the result of inhibition by the substrate, a high mineral content or poor homogenization.

Fixed variables		
X1	=	0.000
X2	=	0.000
X4	=	0.000

Response Y2		
1	:	58.0
2	:	59.3
3	:	60.6
4	:	61.9
5	:	63.2
6	:	64.5
7	:	65.9
8	:	67.2
9	:	68.5
10	:	69.8
11	:	71.1
R	=	1.000
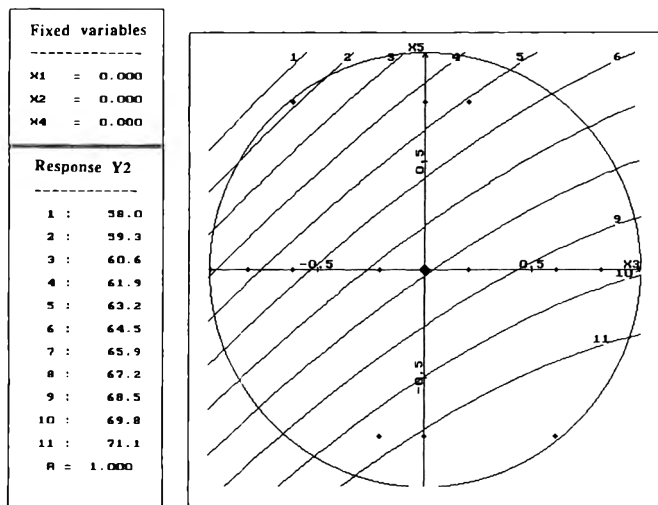
Fig. 5—Response surface contours for protein yield on the supernatant (Y2) as a function of enzyme concentration (X3) and protein concentration (X5). Temperature (X1), pH (X2) and time (X4) were kept at 0 coded levels.

Table 4—Amino acid composition of the raw material (crushed bones) and of hydrolysate powder (g.16g $^{-1}$ N)

Amino acids	Meat bone protein HCl 6N 110°C 24 hr hydrolysis	HCl 6N 110°C 24 hr hydrolysis	Hydrolysate powder methane-sulfonic hydrolysis	Free amino acid
Alanine	7.44	7.11	6.97	0.22
Arginine	6.96	6.84	5.53	0.04
Aspartic acid	6.52	6.41	6.64	0.04
1/2 cystine	1.21	0.19	0.52	0.00
Glutamic acid	11.52	11.00	11.38	0.26
Glycine	14.16	13.33	13.13	0.16
Histidine	3.01	2.05	2.14	0.03
Hydroxyproline	6.98	6.44	5.36	0.00
Isoleucine	2.10	1.79	1.04	0.03
Leucine	5.24	5.01	4.47	0.07
Lysine	6.93	4.62	3.98	0.06
Methionine	0.55	0.04	1.18	0.06
Ornithine	0.28	0.07	0.14	0.03
Phenylalanine	2.81	2.08	2.07	0.04
Proline	8.28	8.01	7.58	0.06
Serine	3.58	3.41	3.47	0.06
Threonine	3.42	3.35	3.12	0.03
Tyrosine	1.78	1.41	1.43	0.02
Valine	3.18	2.45	2.40	0.03
Tryptophan	nm*	nm*	1.84	nm*

*(not measured)

^{a} The composition of essential amino acids in the food grade protein powder was carried out in Experiment 31 (experimental domain center). Each result is the average of two observations.

Fig. 6—Optimal path for protein yield response maximization (Y1) in coded variables. Temperature (X1), pH (X2), enzyme concentration (X3), time (X4), protein concentration (X5).
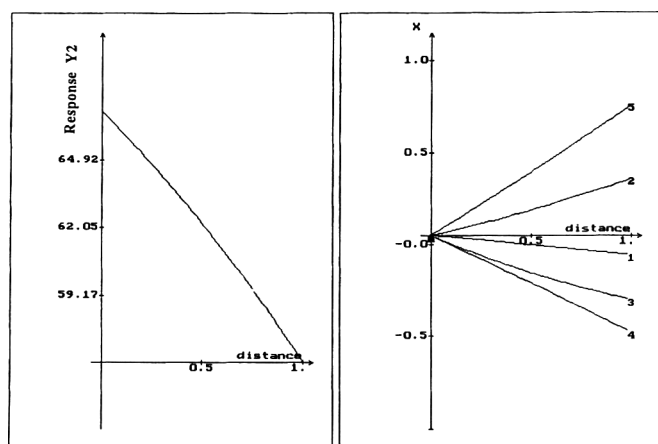
Fig. 7—Optimal path for supernatant protein yield response minimization (Y2) in coded variables. Temperature (X1), pH (X2), enzyme concentration (X3), time (X4), protein concentration (X5).

Considering economic constraints, optimal conditions were established according to the 5 monitoring factors and were shown to maximize yield (Fig. 6), and to minimize the supernatant fraction (response Y2) (Fig. 7). Protein yields from the enzymatic treatment varied from 60–80% of extractable protein, thus limiting production of bitter-tasting fragments. Starting from the center of the experimental domain, the optimal pathway (Figs. 6 and 7) indicates that optimization of yield should be obtained by increasing time and enzyme amount. The results of temperature optimization showed that hydrolysates obtained between 52 and 55°C led to the greatest yield. Enzymatic hydrolyses have been shown to be valuable. The amino acid profile of the substrate and the hydrolysates at the center point were compared (Table 4). There was no major loss of any amino acid. The product was characterized by a high content of important amino acids such as alanine, arginine, aspartic acid and leucine. The hydrolysate was rich in glutamic acid, glycine, hydroxyproline and proline, thus confirming the observation that Neutrase® solubilized the connective tissue collagen. Response surface methodology permitted a predictive experimental model to be developed. The resulting quadratic polynomial equation proved valuable through the initial Doehlert network. The results will enable development of large-scale experiments through characterization of important parameters. The hydrolysates were freeze-dried and stored at −30°C in order to further study their functional and nutritional properties.

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Pyridinoline Cross-links in Bovine Muscle Collagen

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ABSTRACT

Development of muscle collagen cross-linkage was investigated by determining hydroxylysylpyridinoline and lysylpyridinoline contents of longissimus dorsi, semitendinosus and extensor carpi ulnaris muscles. These were removed from male, female and castrated German Simmental cattle (150–620 days old) fattened on different energy levels and related to possible influences of sex, feeding intensity and type of muscle. In intramuscular collagen, an age-related increase in pyridinoline content was found. Cross-link formation was also influenced by sex and feeding intensity. Epimysia exhibited differences in pyridinoline content which were probably due to differences in physical strain of the muscles.

Key Words: meat, collagen, pyridinoline cross-links, epimysium, tenderness

INTRODUCTION

COLLAGEN as the main component of muscle connective tissue is important for the quality of meat. High collagen content affects meat tenderness as well as the biological value of meat protein. Little correlation has been reported between collagen content and meat quality. Meat from young and old animals differs distinctly in tenderness, although their muscle collagen contents differ only slightly (Augustini and Temisan, 1985).

Differences in toughness are caused by maturation processes in connective tissues. These lead to stabilization of the collagen fiber network in adolescent animals through multivalent cross-linking molecules (Bailey, 1984). Stabilization of mature collagen is manifested by decreased solubility (Cross et al., 1984; Augustini and Temisan, 1985), increased mechanical stability (Bernal and Stanley, 1987) and higher resistance to enzymatic reactions (Weiss, 1976). The high stability against degradation by proteolytic enzymes diminishes the value of collagen. Moreover, proteolytic resistance causes preservation of mechanical stability of the collagen fiber upon enzymatic protein degradation during meat maturation. Therefore, intramuscular collagen must be regarded as a primary factor contributing to toughness of meat.

Several compounds postulated to be mature cross-links have been isolated from connective tissues rich in collagen. However, the contents of such compounds did not correspond to age-related stabilization of collagen (Shimokomaki et al., 1972; Robins et al., 1973; Fujii and Tanzer, 1974; Moriguchi and Fujimoto, 1978). Exceptions were the trivalent 3-hydroxypyridinium cross-links hydroxylysylpyridinoline (HP) and lysylpyridinoline (LP) (Fig. 1). For several connective tissues with abundant collagen the content of pyridinolines increased with advancing age (Moriguchi and Fujimoto, 1978; Nakano et al., 1983; Shikata et al., 1985; Eyre, 1987). The pyridinolines connect three collagen molecules, which leads to stabilization of the collagen network. Concerning meat quality, not only collagen content but also age-related cross-linkages must be considered. Because of the sparse occurrence of pyridinolines in collagen,

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it is difficult to determine their amounts. Therefore, knowledge about pyridinolines in intramuscular connective tissue (IMCT) is limited. Our objective was to carry out an extensive determination of the pyridinoline content in bovine muscle with small amounts of connective tissue.

MATERIALS & METHODS

Animal tissues

Muscles were obtained from German Simmental cattle, female, male and castrated, and 150–620 days old. The animals differed in feeding intensity. Half the bulls (Bh) were high-energy fattened by feeding ad libitum maize corn silage and 1.8 kg/day concentrate (high feeding intensity). The other bulls (Bl) were restrictively fed maize silage and 1.0 kg concentrate (low feeding intensity). Heifers (H) and steers (S) were given high-energy-level rations. More details concerning fattening of the animals were reported (Schwarz et al., 1992). Genetic scattering was minimized by the sampling of animals that were all half-brothers and -sisters. A total of 57 animals were raised in this experiment. (Table 1).

At a live weight of 200 kg (beginning of fattening period), 350 kg, 425 kg (only heifers), 500 kg, 575 kg and 650 kg (both bulls and steers),
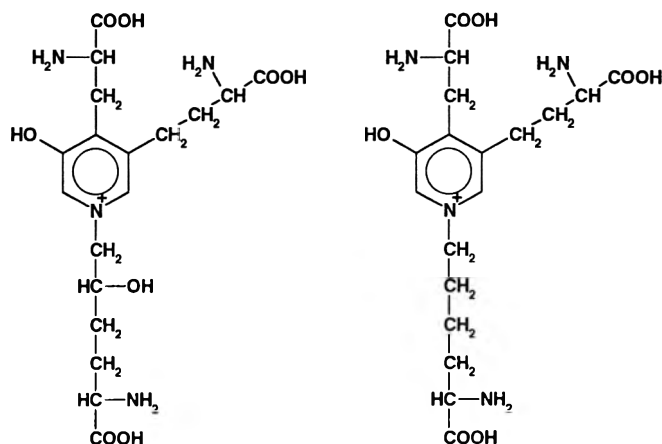
Hydroxylysylpyridinoline (HP)   Lysylpyridinoline (LP)

Fig. 1—Structures of the 3-hydroxypyridinium cross-links hydroxylysylpyridinoline (HP) and lysylpyridinoline (LP).

Table 1—Experimental design

	Low feeding intensity Bull	High feeding intensity		
		Bull	Heifer	Steer
Average daily gain [g]	870	1200	970	980
Slaughter weights [kg]	Age [days]			
200	157 ± 4	157 ± 4	173 ± 13	147 ± 5
350	312 ± 5	261 ± 4	308 ± 4	269 ± 5
425	—	—	382 ± 9	—
500	474 ± 12	368 ± 6	500 ± 20	486 ± 19
575	583 ± 26	509 ± 37	—	536 ± 16
650	618 ± 37	546 ± 34	—	600 ± 38

Table 2—Average collagen contents of bovine musculus extensor carpi ulnaris (ECU) connective tissues (n=3)

| Category | Slaughter weight [kg] | Intramuscular connective tissue | | Epimysium |
| | | Collagen in % of dry weight | | |
		Muscle	Isolate after sieving	Collagen in % of dry weight
Bull, low feeding intensity	200	5.26±0.1	48.8±2.1	56.8±2.2
	350	7.13±0.3	57.2±0.4	78.0±1.3
	500	6.66±0.3	63.7±1.3	87.9±3.8
	575	4.57±0.2	34.5±0.8	83.0±1.5
	650	4.31±0.2	30.0±0.9	69.4±1.8
Bull, high feeding intensity	200	5.26±0.1	48.8±2.1	56.8±2.2
	350	5.34±0.2	47.8±1.3	43.3±0.7
	500	4.99±0.1	44.2±1.5	55.1±1.4
	575	6.20±0.2	38.0±0.9	67.6±1.3
	650	5.46±0.2	32.7±2.6	83.8±2.7
Heifer	200	4.52±0.3	47.1±1.8	53.7±1.4
	350	2.76±0.2	16.9±0.8	60.0±1.0
	425	4.31±0.1	38.5±1.1	70.3±1.6
	500	4.51±0.2	19.2±0.8	85.8±1.9
Steer	200	4.98±0.2	43.5±1.3	66.0±1.3
	350	2.30±0.1	28.2±1.1	66.8±1.2
	500	4.69±0.2	56.2±1.2	78.3±2.1
	575	5.73±0.3	39.4±1.4	73.5±1.6
	650	4.83±0.2	41.7±1.1	68.2±1.6

Table 4—Average collagen contents of bovine musculus semitendinosus (ST) connective tissues (n=3)

| Category | Slaughter weight [kg] | Intramuscular connective tissue | | Epimysium |
| | | Collagen in % of dry weight | | |
		Muscle	Isolate after sieving	Collagen in % of dry weight
Bull, low feeding intensity	200	2.98±0.3	30.1±0.9	27.6±0.7
	350	5.00±0.1	42.9±2.2	55.2±1.6
	500	5.23±0.2	14.7±1.1	39.6±2.1
	575	4.06±0.2	22.5±2.0	41.3±1.2
	650	4.51±0.2	16.5±0.9	41.8±2.2
Bull, high feeding intensity	200	2.98±0.3	30.1±0.9	27.6±0.7
	350	4.05±0.3	31.5±1.6	21.4±1.1
	500	4.50±0.3	42.2±1.7	30.9±1.6
	575	3.58±0.1	17.9±2.0	25.7±1.9
	650	3.78±0.2	10.4±0.9	34.4±2.0
Heifer	200	2.08±0.3	33.7±2.2	22.4±1.9
	350	2.20±0.1	5.05±0.8	14.5±1.9
	425	3.04±0.3	24.1±1.4	26.1±1.3
	500	2.88±0.3	19.7±2.1	28.7±0.5
Steer	200	3.45±0.4	25.8±1.3	31.1±2.4
	350	1.71±0.2	11.7±0.6	31.8±1.0
	500	2.13±0.4	34.9±1.8	46.4±1.8
	575	2.79±0.2	17.2±1.1	30.3±1.5
	650	3.04±0.1	16.6±1.1	22.5±1.4

Table 3—Average collagen contents of bovine musculus longissimus dorsi (LD) connective tissues (n=3)

| Category | Slaughter weight [kg] | Intramuscular connective tissue | | Epimysium |
| | | Collagen in % of dry weight | | |
		Muscle	Isolate after sieving	Collagen in % of dry weight
Bull, low feeding intensity	200	2.04±0.2	27.9±1.4	55.6±2.4
	350	1.81±0.2	26.6±0.9	59.4±1.5
	500	2.69±0.2	17.9±1.2	55.7±0.8
	575	2.71±0.2	18.2±1.0	32.6±2.0
	650	2.47±0.1	22.6±0.8	61.3±2.6
Bull, high feeding intensity	200	2.04±0.2	27.9±1.4	55.6±2.4
	350	2.26±0.1	23.4±2.4	60.7±1.2
	500	2.63±0.1	36.0±1.9	58.8±2.0
	575	2.33±0.2	34.0±1.0	51.4±1.7
	650	2.85±0.2	19.1±1.2	40.0±2.1
Heifer	200	1.80±0.2	32.8±1.7	65.5±1.5
	350	1.77±0.1	4.02±0.5	54.4±1.5
	425	1.72±0.1	14.6±0.9	68.5±2.3
	500	1.73±0.2	22.4±2.2	49.0±1.3
Steer	200	1.96±0.1	32.1±1.9	93.1±1.5
	350	1.03±0.1	3.55±0.9	67.3±2.9
	500	2.30±0.1	31.0±1.8	59.9±1.2
	575	1.81±0.2	33.5±2.1	65.1±1.5
	650	1.71±0.2	60.0±2.3	53.5±2.7

animals were slaughtered. For these investigations three animals of each slaughter point and feeding intensity were chosen. Three muscles differing in collagen content were collected from each animal: longissimus dorsi (LD), semitendinosus (ST) and extensor carpi ulnaris (ECU). A slice of the middle piece of each muscle (~ 250 g) was frozen immediately after slaughtering, vacuum-sealed, and stored at −30°C until analysis for pyridinolines.

Analytical procedure

For isolation of IMCT (Möller et al., 1993) muscle samples were freed from outer fat layers and coarse tendon. The connective tissue covering the muscle (epimysium) was dissected and analyzed separately. After lyophilization, the epimysium was ready for hydrolysis and the determination of pyridinolines omitting the isolation procedure.

About 100g of remaining muscle was homogenized with a meat chopper. Samples (15g) of freeze-dried muscle were blended in a high-speed analytical mill (20,000 rpm) for 1 min while cooling the mill at 4°C. The fibrous IMCT was separated from pulverized muscle protein by

passing the blended muscle sample through a sieve with 160-μm^2 holes with agitation for 10 min on a mechanical shaker. The material left on the sieve was washed and sieved in acetone. After drying at 40°C, blending and sieving were repeated. The isolated IMCT remaining on the sieve was then hydrolyzed in 6 M HCl for 4 hr at 145°C (Roach and Gehrke, 1970). Collagen contents were determined by hydroxyproline analysis before and after isolation.

The first step for determination of pyridinolines (Steinhart et al., 1994) was a partial purification of the crude hydrolysates by cation-exchange chromatography on a phosphocellulose column (H$^+$-form, 2.6 × 30 cm). After injection of the hydrolysate, the column was washed with 450 mL of 0.1M HCl to remove the bulk of the amino acids. Pyridinolines were eluted with 100 mL of 0.5M HCl. The flow rate was 2 mL/min. The pyridinoline fraction was evaporated, the residue taken up in 0.01M HCl and diluted with the same volume of eluent B. Pyridinolines were determined by ion-paired reversed-phase HPLC with sodium octanesulfonic acid as the ion-pairing agent. For separation a reversed-phase Nucleosil 120 3C$_{18}$ column (3 μm, 12.4 cm × 4 mm) was used. The HPLC gradient system consisted of two eluents: A (5g sodium dihydrogen phosphate monohydrate, 1.17g sodium octanesulfonic acid monohydrate, 1 mL of 85% phosphoric acid/L) and B (75% eluent A and 25% acetonitrile (v/v)). The elution gradient program started at 60% A/40% B at time 0, followed by linear gradients to 40% A/60% B for 1 min and then to 15% A/85% B in 14 min, with flow rate 1 mL/min. Pyridinolines were detected by measuring fluorescence (λ_{ex} = 295 nm, λ_{em} = 395 nm). Retention times were HP 9.5 min and LP 10.5 min.

Statistical methods

The unpaired Student's t-test was used to determine statistical significance of differences at $p \leq 0.05$.

RESULTS

THE COLLAGEN CONTENTS of epimysium, muscle and isolated material from three different muscles were compared (Tables 2–4). The means included three determinations. There was a decrease in muscle collagen content in the dry matter from ECU, with an overall average of 4.9 ± 1.1%, to ST, with an overall average of 3.3 ± 1.2%, to LD, with an overall average of 2.1 ± 0.5%. Amounts of connective tissue in muscles of steers and heifers were generally lower (p < 0.05) than those in muscles of bulls Bh and Bl. Collagen in the isolated material ranged from 20% to 45% depending on type of muscle. Epimysial connective tissue of LD and ECU showed similar collagen contents of 55% to 70% (relative to dry weight). Collagen content in ST (25–

Table 5—Contents of lysylpyridinoline (LP) and hydroxylysylpyridinoline (HP) in bovine intramuscular connective tissue (moles/mol collagen)

Category	Slaughter weights [kg]	Musculus extensor carpi ulnaris			Musculus longissimus dorsi			Musculus semitendinosus		
		LP	HP	HP+LP	LP	HP	HP+LP	LP	HP	HP+LP
Bull, low feeding intensity	200	0.003	0.045	0.048±0.015	0.000	0.075	0.075±0.022	0.000	0.091	0.091±0.025
	350	0.007	0.084	0.091±0.033	0.000	0.114	0.114±0.015	0.007	0.094	0.101±0.018
	500	0.006	0.099	0.105±0.016	0.007	0.110	0.117±0.025	0.016	0.120	0.136±0.023
	575	0.000	0.118	0.118±0.032	0.005	0.126	0.131±0.024	0.012	0.125	0.137±0.013
	650	0.000	0.130	0.130±0.023	0.013	0.158	0.171±0.021	0.022	0.201	0.223±0.032
Bull, high feeding intensity	200	0.003	0.045	0.048±0.015	0.000	0.075	0.075±0.022	0.000	0.091	0.091±0.025
	350	0.006	0.079	0.085±0.020	0.006	0.096	0.102±0.029	0.011	0.113	0.124±0.023
	500	0.006	0.094	0.100±0.019	0.007	0.114	0.121±0.024	0.006	0.095	0.101±0.030
	575	0.013	0.116	0.129±0.013	0.016	0.158	0.174±0.021	0.011	0.133	0.144±0.038
	650	0.006	0.121	0.127±0.025	0.019	0.164	0.183±0.029	0.023	0.220	0.244±0.039
Heifer	200	0.000	0.042	0.042±0.022	0.000	0.097	0.097±0.020	0.009	0.112	0.121±0.017
	350	0.000	0.084	0.084±0.017	0.000	0.142	0.142±0.024	0.000	0.165	0.165±0.024
	425	0.014	0.121	0.135±0.014	0.000	0.190	0.190±0.024	0.018	0.183	0.201±0.030
	500	0.018	0.142	0.160±0.032	0.010	0.142	0.152±0.017	0.020	0.158	0.178±0.036
Steer	200	0.004	0.058	0.062±0.029	0.000	0.071	0.071±0.025	0.000	0.093	0.093±0.025
	350	0.006	0.058	0.064±0.024	0.005	0.066	0 071±0.011	0.008	0.098	0.106±0.013
	500	0.004	0.072	0.076±0.028	0.009	0.139	0 148±0.023	0.000	0.120	0.120±0.029
	575	0.000	0.109	0.109±0.017	0.015	0.140	0 155±0.036	0.014	0.125	0.139±0.029
	650	0.010	0.162	0.172±0.026	0.013	0.177	0 190±0.042	0.019	0.156	0.175±0.023

Table 6—Contents of lysylpyridinoline (LP) and hydroxylysylpyridinoline (HP) in bovine epimysial connective tissue (moles/mole collagen)

Category	Slaughter weights [kg]	Musculus extensor carpi ulnaris			Musculus longissimus dorsi			Musculus semitendinosus		
		LP	HP	HP+LP	LP	HP	HP+LP	LP	HP	HP+LP
Bull, low feeding intensity	200	0.005	0.105	0.110±0.034	0.001	0.012	0.013±0.007	0.005	0.074	0.079±0.013
	350	0.005	0.125	0.130±0.025	0.003	0.018	0.021±0.008	0.000	0.089	0.089±0.010
	500	0.000	0.179	0.179±0.019	0.002	0.017	0.019±0.006	0.009	0.095	0.104±0.020
	575	0.000	0.155	0.155±0.022	0.003	0.017	0.020±0.010	0.008	0.104	0.112±0.027
	650	0.000	0.228	0.228±0.027	0.003	0.026	0.029±0.008	0.009	0.104	0.113±0.012
Bull, high feeding intensity	200	0.005	0.105	0.110±0.034	0.001	0.012	0.013±0.007	0.005	0.074	0.079±0.013
	350	0.005	0.172	0.177±0.029	0.002	0.027	0.029±0.005	0.008	0.119	0.127±0.014
	500	0.000	0.171	0.171±0.040	0.002	0.034	0.036±0.011	0.007	0.081	0.088±0.017
	575	0.000	0.196	0.196±0.033	0.002	0.029	0.031±0.005	0.018	0.133	0.151±0.014
	650	0.000	0.273	0.273±0.036	0.003	0.033	0.036±0.014	0.015	0.147	0.162±0.022
Heifer	200	0.003	0.075	0.075±0.010	0.000	0.015	0.015±0.007	0.007	0.094	0.101±0.031
	350	0.000	0.178	0.178±0.039	0.002	0.014	0.016±0.002	0.006	0.137	0.143±0.024
	425	0.003	0.123	0.126±0.025	0.001	0.019	0.020±0.003	0.006	0.132	0.138±0.038
	500	0.003	0.086	0.089±0.021	0.003	0.024	0.027±0.005	0.006	0.151	0.157±0.027
Steer	200	0.000	0.094	0.094±0.012	0.001	0.008	0.009±0.006	0.010	0.109	0.119±0.013
	350	0.000	0.121	0.121±0.035	0.002	0.010	0.012±0.006	0.008	0.118	0.126±0.028
	500	0.000	0.237	0.237±0.047	0.002	0.015	0.017±0.003	0.000	0.173	0.173±0.038
	575	0.000	0.210	0.210±0.041	0.005	0.012	0.017±0.003	0.003	0.175	0.178±0.040
	650	0.000	0.275	0.275±0.040	0.003	0.022	0.025±0.003	0.019	0.167	0.186±0.023

40%), a muscle with higher proportions of elastin, was significantly lower (p < 0.01).

Generally, the collagen contents of connective tissues were sufficient to analyze HP and LP. As in other collagenous tissues, HP was the dominant component of pyridinolines in epimysia and in IMCT's. LP formed only maximally 5–10% of total pyridinolines (Tables 5, 6), which confirmed the results of Eyre (1987) and Steinhart et al. (1994).

The results for pyridinoline content in the tissues were summarized and compared for each fattening category (Fig. 2 and 3) to point out differences in cross-linkage as related to sex and feeding intensity. An age-related increase in total pyridinoline content was obvious in all three types of muscle, in epimysial connective tissue (p < 0.05) as well as in IMCT (p < 0.01). Intramuscular collagen of ECU showed lower pyridinoline contents compared to LD and ST (p < 0.05) (Fig. 2).

Differences in pyridinoline content were found in epimysial samples (Fig. 3). Conspicuously low pyridinoline contents occurred in epimysia of LD. During the examination period, contents rose from 0.009 ± 0.006 to 0.036 ± 0.014 mole pyridinoline/mole collagen. ST epimysia pyridinoline contents ranged from 0.079 ± 0.013 to 0.186 ± 0.023 mole pyridinoline/mole collagen, attaining values similar to those found in IMCT. The highest pyridinoline contents, increasing from 0.075 ± 0.010 to 0.275 ± 0.040 mole pyridinoline/mole collagen, were found in ECU epimysia. These differences in cross-linkage

of epimysial collagen in all fattening categories were obviously specific for each type of muscle.

Comparing results for the different fattening categories, intramuscular collagen of heifers showed higher pyridinoline contents compared to animals of the same age from other categories (p < 0.05). Also note that bulls fattened on low-energy level had a lower degree of cross-linkage in collagenous connective tissue compared to same-age counterparts fattened on high-energy level (p < 0.05) (Fig. 4).

DISCUSSION

IN ALL TISSUES examined, pyridinolines were detected. In accordance with Nakano et al. (1983) and Eyre et al. (1984), the amounts of total pyridinolines as the sum of HP and LP were related to collagen content. The total pyridinoline content in intramuscular collagen varied from 0.05 to 0.28 mole/mole collagen, depending on age. Compared to other connective tissues, cross-linkage of muscle collagen was smaller. Eyre (1987) found pyridinoline contents in connective tissues ranging from 0.3 mole/mole collagen in bones up to 3.0 mole/mole collagen in intervertebral disks. Each pyridinoline molecule connects three collagen molecules, thus a molar ratio of 0.3 indicates a very high degree of cross-linkage in the collagen network.

Several researchers have reported an increase in pyridinoline content with age in various connective tissues abundant in col-

Fig. 2—Pyridinoline contents in intramuscular collagen of bovine muscles. □ musculus extensor carpi ulnaris (ECU); △ musculus longissimus dorsi (LD); ● m usculus semitendinosus (ST); Bl = bull, low feeding intensity; Bh = bull, high feeding intensity; H = heifer; S = steer.

Fig. 3—Pyridinoline contents in epimysial collagen of bovine muscles. □ musculus extensor carpi ulnaris (ECU); △ musculus longissimus dorsi (LD); ● musculus semitendinosus (ST); Bl = bull, low feeding intensity; Bh = bull, high feeding intensity; H = heifer; S = steer.

lagen (Moriguchi and Fujimoto, 1978; Nakano et al., 1983; Shikata et al., 1985). This increase with advancing age was also ascertained in bovine intramuscular collagen. The pyridinoline content in IMCT of musculus sternomandibularis (STERN) from older milk cows (Steinhart et al., 1994) was compared with that of ECU from heifers in our results (Fig. 5). The animals were of the same gender and both muscles had nearly equivalent amounts of connective tissue. Thus, results were combined to reveal tendencies. As expected, the pyridinoline content of intramuscular collagen of heifers to 18 mo was smaller than with older animals. During the first months an increase in pyridinoline content was much faster than in the period following physiological maturity, when cross-link formation proceeded at a noticeably slower rate. Similar changes have been reported by Moriguchi and Fujimoto (1978) for pyridinoline content in rat cartilage collagen. The continuous increase in pyridinoline content after reaching physiological maturity indicated that pyridinolines in intramuscular bovine collagen also should be considered as "mature" cross-linking molecules. An increase in pyridinoline content is accompanied by diminution of labile cross-links and by enhancement in mechanical stability of collagen fibers. Moriguchi and Fujimoto (1978) indicated that formation of cross-links was an essential step of growth in order to obtain and uphold mechanical strength of the collagen fibers.

The extent of cross-linkage seemed to be closely related to the physiological functions of the tissues. Although the muscles examined (ST, ECU and LD) had different physiological functions, their pyridinoline contents in the intramuscular collagen were very similar compared to the content in epimysial collagen. LD was the largest of the three muscles and supports carrying capacity of the vertebral column. It contributes essentially to the elasticity of the body during forward and backward movement. The two other muscles are active and heavily stressed muscles of the motor system. ECU, with more connective tissue, is less voluminous than ST which is quite fleshy. The main function of IMCT is to prevent injury and overstraining of contractile elements of muscle fibers. Obviously, the higher strain of ST and ECU is mainly equalized by the content of IMCT and not by the degree of cross-linkage. The different functions of muscles are distinctly reflected in their cross-linkage of epimysial collagen.

Unlike intramuscular collagen, epimysium is more responsible for efficient transmission of contractile power of the muscular elements via tendons to the skeleton. It is more likely that the low pyridinoline content of LD epimysium is related to its function, which requires a higher elasticity of surrounding connective tissue layers. In contrast to LD, ST and ECU are more stabilized for a better support of motion through more extensive epimysium cross-linkages. Because of the smaller size of ECU, its epimysium has a more tendonlike function. This explains its epimysium having the highest pyridinoline content. A correlation between function and cross-linkage of connective tissue was also reported by Eyre et al. 1984. In tendons and ligament they found differing pyridinoline contents and associated these with physical strains of the tissues.

The gender of the animals had little influence on muscle collagen cross-linkage. The hormone status of the animals had a distinctly greater effect on the content of muscle connective tissue. Independent of feeding intensity, bull muscles contained larger amounts of collagen than steer and heifer muscles. This was in agreement with reported results (Prost et al., 1975; Jeremiah and Martin, 1982; Seideman, 1986). Nevertheless, in many samples, pyridinoline contents of heifers, compared to other animals, were higher, especially in the center region of each age period. At the end of the investigation period differences no longer occurred (Fig. 2). Heifers attain physiological maturity faster than steers and bulls, so that cross-linkage during the first months of life is initially faster but reaches a constant rate. This resulted in an equalization of pyridinoline contents of different sexes at the end of the age period.
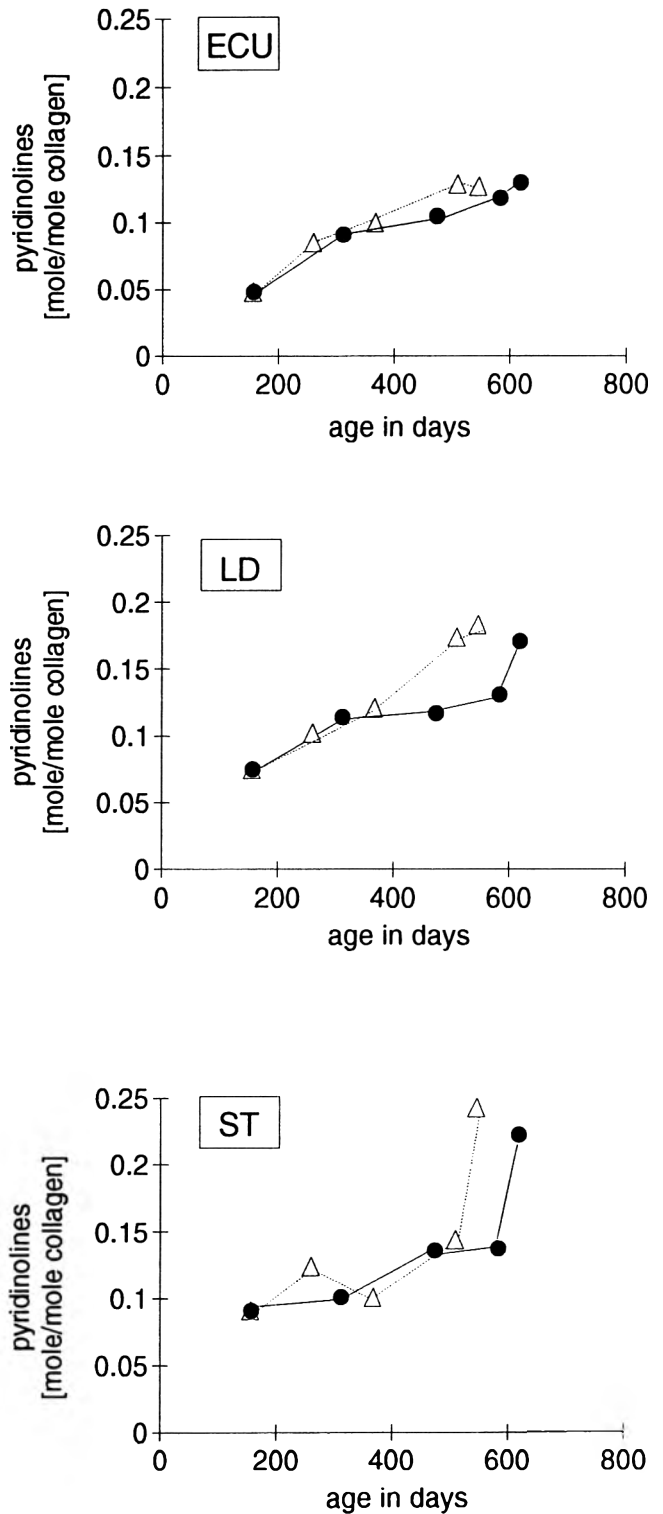
Fig. 4—Influence of feeding intensity on pyridinoline content. △ bull, high feeding intensity; ● bull, low feeding intensity; ECU=musculus extensor carpi ulnaris; LD=musculus longissimus dorsi; musculus semitendinosis.

Low-energy-level fattening apparently decelerated physiological aging processes. The pyridinoline content was lower in muscles of bulls fattened on low-energy level compared to their counterparts fattened on high-energy level. This indicated that, with low-energy-level fattening, the high collagen turnover at the beginning of growth was preserved for a longer time.

From our results, increased toughness of meat with age is not caused by an increase in collagen content. This is in agreement
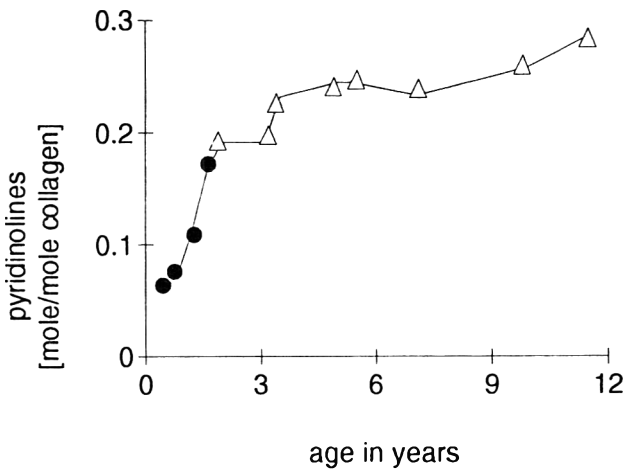
Fig. 5—Pyridinoline contents in intramuscular collagen of bovine △ musculus sternomandibularis (STERN) and ● musculus extensor carpi ulnaris (ECU). (Steinhart et al., 1994)

with the previous results (Cross et al., 1984; Augustini and Temisan, 1985). More likely the stabilization of collagen through cross-linkage with advancing age leads to a lessening of meat tenderness (Bailey, 1984). The rise in pyridinoline content of muscle collagen probably contributes essentially to this decrease.

Age, sex and feeding intensity influence meat tenderness. For a general overall description of their effects we can characterize tenderness by measurement of shear force or by sensory characteristics. This will show the results of changes in tenderness caused by these factors, but will not explain how such changes came about. With the determination of pyridinolines it is possible to describe changes in tenderness at the physiological and biochemical levels. Because of their thermal and chemical stability the pyridinolines are insensitive to other influences affecting meat tenderness. The results of shear force measurement or analysis by sensory panels commonly used for description of tenderness may be obscured by cold-shortening or other effects from mistreatment during slaughter or storage. The determination of pyridinolines is an objective method for characterization of tenderness which derives from the meat and not from possible changes by processing treatments.

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Ms received 12/12/94; revised 3/27/95; accepted 4/17/95.

Fatty Acid Content of Supercritical Carbon Dioxide Extracted Fractions of Beef Fat

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ABSTRACT

Utilization of beef fat might be improved via production of fractions with specific functional properties. Beef fat was fractionated using supercritical carbon dioxide at 40°C and pressures from 10.3–27.6 MPa. Extractions followed by one or two stage separation were collected into glass vials at atmospheric pressure and temperature. Resultant extracts were analyzed for fatty acid content by gas chromatography (GC). A significant difference in concentration due to extraction pressure for 16 straight chain fatty acids, 6 branched chain fatty acids, and 7 unknowns was found. Total fatty acids (mg/g extract) increased as pressure increased. Monounsaturated and polyunsaturated fatty acids increased with increasing pressure and solvent density as shown by the saturate/unsaturate, saturate/monounsaturate, and saturate/polyunsaturate ratios.

Key Words: beef, fatty acids, supercritical CO_2, tallow, unsaturated

INTRODUCTION

WORLDWIDE PRODUCTION of tallow and grease is over 6 million metric tons. Annual production of edible tallow in the U.S. is >0.5 million metric tons. Health concerns about saturated fats and cholesterol have decreased the utilization of this by-product. Utilization of beef fat might increase if it could be separated into various components which could be used for specific food applications. Altering fatty acid ratios to provide specific compositions or result in a more "healthy" image may also be possible.

Supercritical fluids are used commercially to extract caffeine from coffee and tea, essential oils for fragrances, and hop oil. Supercritical fluids are useful to extract oils from oil seeds (Stahl et al., 1980; Bulley et al., 1984; Friedrich and Pryde, 1984; Temelli, 1992) and cholesterol from milk (Bradley, 1989). Cholesterol and lipids have also been extracted from meat products (Hardardottir and Kinsella, 1988; King et al., 1989, 1993; Chao et al., 1991) and eggs (Froning et al., 1990). Many researchers have studied fractionation of milk fat using supercritical fluids (Kaufmann et al., 1982; Shishikura et al., 1986; Arul et al., 1987; Bhaskar et al., 1993).

Sharp differences occur in the density and solvating power of supercritical fluids depending on temperature and pressure used. At high pressures and temperatures, little or no selectivity of extraction occurs, rather, an extraction of a majority of the nonpolar compounds has been observed. Conversely, at pressures and temperatures closer to the critical point, the density and solvating properties of supercritical fluids vary. These variations affect efficiency of separation and extraction (Brogle, 1982; Allada, 1984).

Few researchers have reported use of supercritical fluids to fractionate lipids associated with beef subcutaneous fat. Our objective was to determine the effect of extraction pressures on the fatty acid composition of resulting beef lipid fractions.

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MATERIALS & METHODS

Materials

Subcutaneous fat was collected from the loins of 12 Angus-Hereford cross steers. Fat was pooled and ground once through a 0.95 cm plate. Portions (50g) were weighed and vacuum-packaged in low permeability bags (Cryovac Corp., Duncan, SC) and overwrapped with polyethylene coated freezer paper. Samples were stored at −10°C and allowed to thaw at refrigeration temperature (2–4°C) prior to use.

Supercritical fluid extraction

A Superpressure Model 46-1341-2 supercritical fluid extractor (Newport Scientific, Jessup, MD) with a 69.0 MPa double and diaphragm compressor was used to fractionate the beef fat (Fig. 1). A 50-g sample, immobilized between two plugs of glass wool, was loaded into a 0.845L (internal volume) stainless steel extraction vessel. A one- or two-stage separation from supercritical carbon dioxide followed the single extraction step (Table 1). For a two-stage separation, a 0.500L stainless steel separation vessel was used between the extraction vessel and the collection vessel. The temperature of both vessels was maintained at 40°C, using heaters wrapped around the outside of the vessels and an internal thermocouple under thermostatic control. All stainless steel transfer lines were insulated to prevent heat loss and precipitation of solubilized material due to temperature drop. Gas was depressurized at room temperature and atmospheric pressure into a 0.500L Pyrex glass flask. Flow was maintained at 15 L/min through the flow indicator and the extraction was terminated after 500L of carbon dioxide gas (measured at room temperature and pressure) were measured on the flow totalizer. Samples were transferred, with a pasteur pipette, into 16 mm × 125 mm Pyrex tubes fitted with teflon lined caps. Tubes were nitrogen-flushed and placed into a freezer at −10°C until fatty acid analysis was performed.

Fatty acid analysis

Fatty acid contents of samples were determined using a modified fatty acid methyl ester method (Morrison and Smith, 1964). A sample (~100 ± 0.01 mg) was weighed into a 16 mm × 125 mm Pyrex tube fitted with a teflon-lined cap. The sample was diluted with 10 mL chloroform-methanol (2:1 v:v) with 0.01% butylated hydroxy toluene (BHT). A sample (1 mL) was transferred using a volumetric pipet to another clean 16 mm × 125 mm Pyrex tube. Boron triflouride (12%) in methanol (2 mL) was added as a catalyst. Pentadecenoic acid (C15:1; 40 µL of 20.8964 µg/mL) in chloroform:methanol (2:1 v:v) was added as internal standard. Tubes were placed into a heating block at 100°C for 30 min. Tubes were removed and 4 mL saturated (26.4%) sodium chloride solution was added to each tube. Optima (Fisher Scientific, Fair Lawn, NJ) high purity hexane (2 mL) was added to extract the methyl esters. Tubes were shaken well and centrifuged for 5 min to aid in separation of the phases. The top hexane layer was transferred to a conical, graduated 5 mL Pyrex tube. The tube was washed with another 2 mL hexane. The hexane portion was evaporated to 1 mL. Sodium sulfate was added to bind any residual water.

A Hewlett Packard 5890 gas chromatograph, equipped with a 30m DB-23 fused silica column (J & W Scientific, Folsom, CA) with internal diameter 260 µm and a film coating thickness of 0.25 µm was used to separate fatty acid methyl esters. Helium carrier gas column flow rate was 1.7 mL/min at a head pressure of 1.75 kg/cm² with a 1:22 split ratio. The injector temperature was maintained at 230°C while the detector was at 240°C. A two step linear temperature program of 150°C to 200°C at 4°C/min and 200°C to 222°C at 10°C/min (total run time of 14.7 min) was used for separation. A flame ionization detector (FID) with nitrogen makeup gas was used for detection. Peak areas were calculated using a Spectra Physics SP4100 computing integrator.
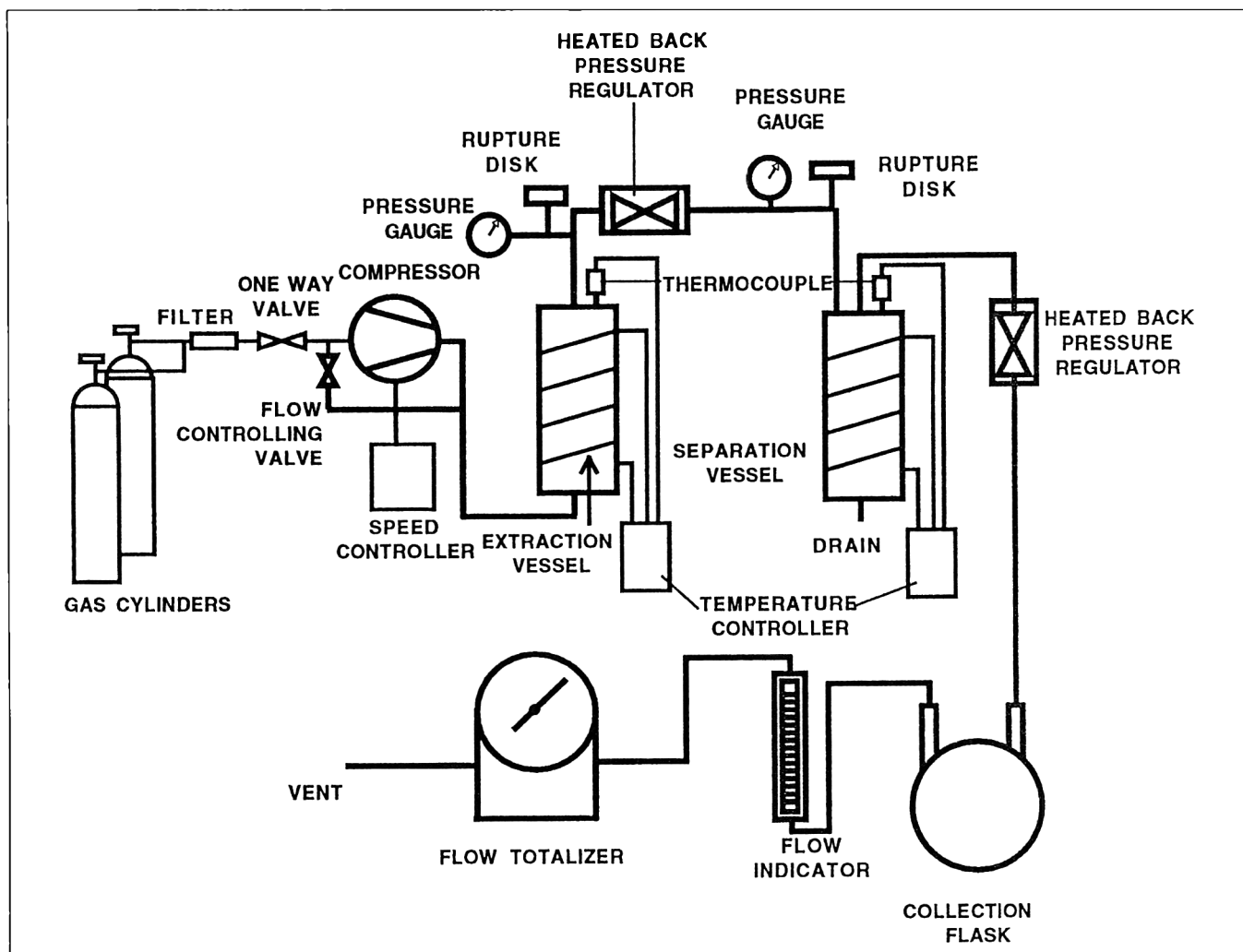
Fig. 1—Schematic diagram of supercritical extractor.

Table 1—Experimental design of extraction pressures

		Separation vessel pressure (MPa)			
		20.7	17.2	13.8	10.3	0ª
Extraction	27.6	X	X	X	X	
vessel	20.7		X	X	X	X
pressure	17.2			X	X	X
(MPa)	13.8				X	X
	10.3					X

ª 0 indicates that separation vessel was not used.

Response factors were calculated by comparing peak areas of known quantities of authentic standards (Nu Check Prep, Inc., Elysian, MN) to the internal standard, pentadecenoic acid.

Mass spectrometry

Fatty acid methyl esters were prepared as described above and a 0.5 μL sample was injected directly onto a 30 m DB-23 capillary column (J & W Scientific, Folsom, CA) with internal diameter 320 μm and a film thickness of 1 μm. The column was held at an initial temperature of 150°C for 2 min and then programmed from 150°C to 200°C at 4°C/min and from 200°C to 230°C at 10°C/min. Methyl esters were analyzed using a Hewlett Packard 5897 gas chromatograph/mass spectrometer with an ionization potential of 70 eV and a scanning range of 400–500 atomic mass units. Peaks were identified by comparing retention times and mass spectra to authentic standards (Matreya, Inc., Pleasant Gap, PA; Nu Check Prep, Inc., Elysian, MN) prepared in an identical fashion.

Experimental design

A range of extraction and separation pressures was used (Table 1). A single extraction, followed by one (atmospheric pressure) or two (separation vessel followed by atmospheric pressure) stage separation from supercritical carbon dioxide was carried out. A wide range of pressures was used from just above the critical pressure at 10.3 MPa to 27.6 MPa. All extractions were performed in duplicate. An unextracted sample of subcutaneous fat was analyzed as a "control" to evaluate the degree of fractionation.

Analysis of variance was calculated for each data set using the general linear model procedure (SAS, 1985). Waller-Duncan k-ratio t-tests were calculated for significantly different main effect means (P ≤ 0.05). Means were considered significantly different if P ≤ 0.05 unless otherwise indicated.

RESULTS & DISCUSSION

Fatty acid analysis

Fatty acid concentrations of extracted samples were compared (Table 2). Only those fatty acids which were positively identified and which were influenced by extraction conditions are reported. Also, only those extraction treatments which resulted in significant fractionation are reported. Samples prepared at extraction vessel:separation vessel (EV:SV) pressures of 27.6:17.2, 20.7:17.2, 27.6:13.8, 20.7:13.8, 20.7:10.3, 20.7:0 and 17.2:0 did not result in significant fractionation of triglycerides as indicated by a lack of statistical differences in fatty acid compositions.

Table 2—Fatty acid content (mg/g extract) of supercritically extracted beef samples

Sample EV p^d	ID^a RT^c / SV p^d	C10:0^b 1.87	C12:0 2.50	C14:0 3.64	C14:1 3.96	i-C15:0 4.06	a-C15:0 4.20	C15:0 4.45	i-C16:0 4.97	C16:0 5.48	i-C16:1 5.59	C16:1 5.79	i-C17:0 6.05
Control		0.66^hi	1.16^hi	36.35^fgh	8.04^h	5.87^fgh	5.76^fgh	9.40^fg	3.32^fg	205.13^fg	1.16^f	26.04^g	4.87^fg
27.6	20.7	1.16^fghi	1.60^fgh	44.50^fg	11.54^fg	6.69^f	6.63^f	10.61^f	4.02^f	243.49^f	0.50^g	35.63^f	6.40^f
17.2	13.8	1.77^f	2.10^f	45.05^f	12.12^f	6.40^fg	6.10^fg	9.34^fg	3.23^fgh	178.90^gh	0.23^gh	28.01^fg	4.27^g
27.6	10.3	1.25^fgh	1.53^fgh	34.79^gh	9.81^fgh	5.31^gh	4.88^gh	7.48^gh	2.70^gh	154.07^gh	0.36^gh	24.72^g	3.83^g
17.2	10.3	1.59^f	1.89^fg	43.62^fg	11.67^fg	6.25^fg	6.10^fg	9.42^fg	3.31^fg	188.14^fgh	0.18^gh	29.21^fg	4.67^g
13.8	10.3	1.18^fghi	1.41^gh	32.55^h	9.09^gh	4.64^h	4.39^h	6.83^h	2.35^h	134.10^h	0.18^gh	22.14^g	3.32^g
13.8	0^e	0.52^i	0.66^i	12.78^i	3.34^i	2.04^i	1.61^i	2.56^i	0.85^i	48.89^i	0.05^h	7.64^h	0.97^h
10.3	0^e	1.43^fg	1.68^fgh	37.35^fgh	10.80^fg	5.73^fgh	5.28^fgh	7.89^gh	2.78^gh	153.71^gh	0.17^gh	25.97^g	3.89^g

EV p^d	ID^a RT / SV p^d	a-C17:0 6.25	C17:0 6.61	C17:1 6.94	C18:0 7.92	trans 9 C18:1 8.08	cis 9 C18:1 8.25	cis 11 C18:1 8.33	C18:2 8.90	C18:3 9.75	C20:0 10.69	FAME SUM TOTAL
Control		6.67^fg	10.26^fg	4.82^gh	111.10^fg	3.11^fg	213.64^fgh	7.64^fg	10.31^gh	1.90^gh	0.53^fg	798.30^fg
27.6	20.7	8.13^f	12.62^f	7.11^f	130.82^f	4.09^f	288.99^f	9.14^f	14.94^f	2.88^f	0.75^f	976.33^f
17.2	13.8	5.30^gh	7.49^gh	4.24^gh	68.30^hi	2.23^gh	160.92^gh	5.13^hij	7.92^gh	1.58^gh	0.19^hij	672.89^gh
27.6	10.3	4.99^gh	7.26^gh	4.47^gh	70.64^hi	2.36^gh	166.84^gh	4.91^ij	7.27^gh	1.72^gh	0.39^ghi	635.97^gh
17.2	10.3	5.85^gh	8.55^gh	5.78^fg	80.34^ghi	2.48^gh	189.82^gh	6.38^ghi	9.36^gh	1.83^gh	0.16^hij	730.60^gh
13.8	10.3	4.13^hi	5.69^hi	3.29^h	54.09^ij	1.87^h	132.34^h	4.12^j	6.91^h	1.24^hi	0.15^hij	538.70^h
13.8	0^e	1.41^i	1.80^i	1.04^i	19.19^j	0.64^i	44.93^i	1.17^f	2.13^i	0.41^i	0.00^j	257.86^i
10.3	0^e	4.81^gh	7.01^gh	4.92^gh	61.16^hi	2.01^gh	157.13^gh	5.13^hij	7.78^gh	1.62^gh	0.13^ij	612.60^gh

[a] ID = Identification by mass spectrometry and retention time.
[b] Compounds identified by number (n) of carbons, followed by degree of unsaturation.
  i = (n-1)-methyl; a = (n-2)-methyl branch of the corresponding n carbon fatty acid.
[c] RT = Retention time.
[d] EV p = Extraction vessel pressure; SV p = Separation vessel pressure.
[e] Separation vessel pressures of 0 indicates that the separation vessel was not used - one stage extraction.
[f-i] Means in the same column with the same letter do not differ significantly (P ≤ 0.05).

The concentration of 32 of the 37 fatty acids was influenced (P ≤ 0.05) by extraction and separation conditions.

Total fatty acids (mg/g extract) increased as pressure increased. The highest extraction pressure, 27.6:20.7 (extraction pressure 27.6 MPa; two stage separation pressure 20.7 MPa and atmospheric pressure), resulted in a higher yield of fatty acids than the control. This was due to low solubility of connective tissue, phospholipids, and other polar compounds in supercritical carbon dioxide which resulted in their remaining in the extraction vessel. As pressure was decreased, the yield of extract and the concentration of fatty acids in the extract decreased. This resulted from a decrease in solvating power of the solvent and an increase in concentration of compounds (e.g., volatiles) with high partial pressures in supercritical fluid (Merkle and Larick, 1994). Monounsaturated and polyunsaturated fatty acid concentration increased as solvent density increased. A change in total fatty acids (mg/g) was observed in the two stage separations from treatments with the same separation pressure and different extraction pressures. For such samples the yield of fatty acids increased with increased extraction pressure. This may be due to a precipitation occurring as lipid-rich supercritical carbon dioxide dropped in density from the higher pressure extraction vessel to the lower pressure separation vessel. Such pressure drop results in a precipitation of material which is no longer soluble in the less dense fluid. As this density difference increases greater amounts of material precipitate, entrapping other high molecular weight material to precipitate with it. This results in a decrease in triglyceride content as indicated by fatty acid concentration (mg/g). This trend (P ≤ 0.10) can be observed by comparing the treatments 27.6:20.7 MPa and 27.6:17.2 MPa, the fatty acid content of the treatment with the higher pressure drop, (27.6:17.2 MPa), was lower than that of the treatment with the lower pressure drop (27.6:20.7 MPa). Concentrations were 767 and 976 mg/g extract, respectively. A similar trend (P ≤ 0.10) was observed when comparing the pair of treatments, 17.2:13.8 MPa and 17.2:10.3 MPa, which had fatty acid concentrations of 672 mg/g and 730 mg/g extract, respectively.

Fatty acid analysis resulted in the identification of 14 saturated, 8 monounsaturated, 2 polyunsaturated, and 13 unknown compounds. Unknowns together comprised a very small fraction of the total fatty acid content (data not shown).

Saturated fatty acids identified in the fractions included: decanoic (C10:0), dodecanoic (C12:0), tridecanoic (C13:0), tetradecanoic (C14:0), 13-methyltetradecanoic (i-C15:0), 12-methyltetradecanoic (a-C15:0), pentadecanoic (C15:0), 14-methylpentadecanoic (i-C16:0), hexadecanoic (C16:0), 15-methylhexadecanoic (i-C17:0), 14-methylhexadecanoic (a-C17:0), heptadecanoic (C17:0), octadecanoic (C18:0), and eicosonoic (C20:0) acid. The major constituents of this class (≈75%) were hexadecanoic and octadecanoic acids. The presence of odd numbered chain and branched chain fatty acids have been previously demonstrated in fat from forage-fed steers (Marmer et al., 1984; Holden, 1985). The saturates comprised the majority of the total fatty acids identified. The 27.6/20.7 MPa treatment resulted in the highest concentration of saturated fatty acids, greater (P ≤ 0.10) than the control. The amount of saturated fatty acids/g decreased as pressure decreased. The trend for total fatty acids followed that of saturated fatty acids, i.e., concentration decreased with decreasing pressure and solvent density.

Monounsaturated fatty acids identified in the fractions included: tetradecenoic (C14:1), 14-methylheptadecenoic (i-C16:1), hexadecenoic (C16:1), heptadecenoic acid (C17:1), trans-9-octadecenoic (trans-9-C18:1), cis-9-octadecenoic (cis-9-C18:1), cis-11-octadecenoic (cis-11-C18:1), and eicosenoic (C20:1) acids. The 27.6:20.7 MPa treatment resulted in the highest concentration (mg/g) of monounsaturated fatty acids. The two-stage separations showed similar results to those observed in the total extraction. At a given separation pressure, increasing the extraction pressure tended to result in an increase in monounsaturated fatty acids (as expressed in mg/g). Single stage separation showed a slight decrease from highest pressure to lowest pressure with the exception of the single stage separation of the 13.8 MPa extraction which was much lower than all other treatments.

Polyunsaturated fatty acids identified included: octadecadienoic (C18:2) and octadecatrienoic (C18:3) acids. The concentration of these fatty acids was similar throughout the treatments with the 27.6/20.7 MPa treatment being higher and the single stage separation, 13.8 MPa, being lower than the control for both acids. There was no difference in polyunsaturated fatty acid composition between the other treatments. This was due to the low concentration of polyunsaturated fatty acids in beef tallow.

Table 3—Summary of fatty acids from supercritical fluid extracted beef tallow

Treatment EV p[b]	SV p[b]	Fatty acid (mg/g)					Fatty acid ratios			
		SAT[a]	MONO[a]	POLY[a]	TOTAL UNSAT	TOTAL FA	SAT/ UNSAT	SAT/ POLY	SAT/ MONO	MONO/ POLY
Control		401.3[de]	264.7[def]	12.2[ef]	276.9[def]	798.3[de]	1.45	32.87[de]	1.52	21.68
27.6	20.7	477.8[d]	357.5[d]	17.8[d]	375.3[d]	976.3[d]	1.27	26.82[f]	1.34	20.06
27.6	17.2	366.6[e]	274.3[de]	13.1[de]	287.5[de]	767.7[de]	1.28	27.97[ef]	1.34	20.93
20.7	17.2	322.2[ef]	233.9[fg]	11.4[ef]	245.3[ef]	679.0[ef]	1.31	28.23[ef]	1.38	20.50
27.6	13.8	326.8[ef]	237.6[fg]	11.3[ef]	249.0[ef]	684.1[ef]	1.31	28.82[ef]	1.38	20.96
20.7	13.8	345.9[ef]	252.8[fg]	12.5[ef]	265.2[ef]	724.3[ef]	1.30	27.76[ef]	1.37	20.29
17.2	13.8	338.8[ef]	213.1[fg]	9.5[ef]	222.7[ef]	672.9[ef]	1.52	35.66[d]	1.59	22.44
27.6	10.3	288.4[ef]	213.8[fg]	9.0[ef]	222.8[ef]	636.0[ef]	1.34	33.31[de]	1.40	23.78
20.7	10.3	340.2[ef]	250.4[fg]	11.9[ef]	262.2[ef]	715.1[ef]	1.30	28.66[ef]	1.36	21.09
17.2	10.3	360.3[ef]	245.8[fg]	11.1[ef]	257.0[ef]	730.6[ef]	1.40	32.23[def]	1.47	21.98
13.8	10.3	255.1[f]	173.3[g]	8.2[f]	181.4[f]	538.7[f]	1.41	31.30[def]	1.47	21.26
20.7	0[c]	323.0[ef]	236.5[fg]	11.4[ef]	247.9[ef]	679.4[ef]	1.30	28.33[ef]	1.37	20.74
17.2	0	336.9[ef]	228.5[fg]	10.7[ef]	239.2[ef]	689.6[ef]	1.41	31.60[def]	1.47	21.44
13.8	0	93.4[g]	59.0[h]	2.5[g]	61.5[g]	257.9[g]	1.52	36.77[d]	1.58	23.22
10.3	0	293.2[f]	206.4[fg]	9.4[ef]	215.7[ef]	612.6[ef]	1.36	31.19[def]	1.42	21.95

[a] SAT = Saturated; MONO = Monounsaturate; POLY = Polyunsaturated; UNSAT = Unsaturated.

[b] EV p = Extraction vessel pressure (MPa); SV p = Separation vessel pressure (MPa).

[c] Separation vessel pressure of 0 indicates that the separation vessel was not used - one stage extraction.

[d-g] Means in the same column with the same letter do not differ significantly ($P \le 0.05$).

Saturated/unsaturated, saturated/monounsaturated, saturated/polyunsaturated, and monounsaturated/polyunsaturated ratios changed as extraction pressures changed (Table 3). Ratios for all comparisons were similar to the control at the low pressures. As pressure increased, these ratios decreased indicating that triglycerides containing the more unsaturated fatty acids were soluble at higher densities.

These data indicated that at pressures nearest the critical point, where carbon dioxide had lowest density, the triglycerides containing the short-chain nonpolar fatty acids were most soluble. These results were similar to those reported by Froning et al. (1990) who demonstrated that supercritical extraction of lipid in egg yolk resulted in an increased saturate/unsaturate ratio in the remaining egg product as extraction pressure was increased. Chao et al. (1991) reported similar results with differential scanning calorimetry. Extracts at higher pressures resulted in higher peaks at lower temperature regions and lower peaks in higher melting regions corresponding to higher polyunsaturated and lower saturated fatty acid composition in those fractions.

The solubility of compounds has been previously correlated with vapor pressures (Wong and Johnston, 1986). Increasing pressure results in increased yield due to a greater solvent capacity (de Filippi, 1982). The optimal fractionation conditions, however, are near the critical temperature and pressure (Brogle, 1982).

CONCLUSIONS

EXTRACTION CONDITIONS affected the fatty acid content of extracts. Within a specific carbon chain length, the extraction of fatty acids was based on the degree of unsaturation. At lower pressures, more saturated fatty acids were extracted. As pressures and density of fluid increased, the amount of unsaturated compounds and degree of unsaturation increased as shown by the saturate/unsaturate and monounsaturate/polyunsaturate ratios. Differences between fatty acid composition of beef fat extracts were obtainable by variation in extraction pressure but no true separations of fatty acids were observed. SFE can be useful in fractionating beef fat. Differences in extracts demonstrate a potential for development of fractions which may be useful to the food industry specific food applications.

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Ms received 5/18/94; revised 2/17/95; accepted 4/6/95.

Consumer Acceptability of Beef Steak Tenderness in the Home and Restaurant

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ABSTRACT

Acceptability of beef steak tenderness in home and restaurant environments was evaluated by 62 consumers. Steaks (loin, ribeye, or bottom and top round) were consumed by each panelist in their home and in a fine dining restaurant. Each consumer rated steaks for tenderness and overall acceptability in the home (over a 2-mo period) and one steak per week (over a 7-wk period) in the restaurant. Acceptability for steaks consumed at home and in the restaurant ranked between 3 (moderately tough) and 4 (slightly tough) on an 8-point scale. The Warner-Bratzler Shear force transition level for beef tenderness acceptability in the home was between 4.6 and 5.0 kg and in the restaurant between 4.3 and 5.2 kg. The unacceptable level of beef steak tenderness was ≤4.3 kg. Results suggested that consumers were more critical of beef tenderness in the home than in restaurants. Consumer responses may have been influenced by receiving free meals in the home or restaurant, as well as by the dining environment.

Key Words: beef, palatability, sensory, shear force, tenderness

INTRODUCTION

TENDERNESS is the most important palatability attribute of meat and a primary determinant of meat quality (Dikeman, 1987). The National Beef Tenderness Survey revealed that beef production practices result in considerable variation in tenderness and an unacceptable percentage of tough meat (Smith et al., 1992). Several factors, such as breeding and feeding conditions, contribute to variations in tenderness (Crouse et al., 1991; Wheeler et al., 1990; Shackelford et al., 1991).

Retailers and restaurateurs have rated tenderness as a top concern (Smith et al., 1992). Meeting the tenderness needs for those markets could lead to more consumer satisfaction, and possibly increased consumption of beef. Establishing a "tenderness transition level" for acceptability in consumer markets (restaurant vs home) could lead to new marketing systems for beef of various qualities. For example, a tenderness value could be placed on a package for sale to restaurants or in the retail case. Knowing what a consumer may perceive as acceptable tenderness in a restaurant as compared with the home has other implications. The industry might establish an acceptability level, based on values given by consumers, at which meat portions would be considered undesirable to eat or would require an additional treatment.

Our objectives were to determine acceptability levels for beef steak tenderness, as assessed by the same group of consumers in restaurant and home settings, and to determine any relationships between Warner Bratzler Shear (WBS) force values and beef tenderness ratings by consumers on paired steaks in both the home and restaurant.

MATERIALS & METHODS

Meat

Sides of beef (22) and loin strips (30) (IMPS ¦ 180) were purchased from the Excel Corporation (Plainview, TX). Individual steaks 2.0 cm thick were cut from the rib, loin, and top and bottom rounds. Steaks averaged 225g. The ribeye and loin strip steaks contained only the longissimus muscle, the top round steak was the semimembranosus and the bottom round steak was the biceps femoris. After sample numbers were assigned, each steak was vacuum packaged in Cryovac B550 bags (Cryovac Division of W.R. Grace & Co., Duncan, SC). The steaks for the home and restaurant study were stored at −20°C for 45 days until all data were collected. Steaks for both the home and restaurant were not significantly different for Warner Bratzler Shear Forces Values although they varied in muscle group. Thus, the tenderness variation for both groups was the same.

Warner-Bratzler shear evaluation

One hundred eighteen steaks (randomly selected from the four primal cuts) were thawed at 2°C for 48 hr. Steaks from each primal cut for shear testing were paired with those to be evaluated by consumers in both the home and restaurant. Steaks were broiled on an Open Health electric broiler (Faberware Company; Bronx, NY) to an internal temperature 70°C. Temperature was monitored with a digital meat thermometer (Taylor, model 9865). Cooked steaks were chilled for 24 hr at 2°C for Warner-Bratzler shear (WBS) force testing. To obtain shear force values, six 1.3 cm diameter cores were removed from each of the chilled steaks, parallel to the muscle fiber orientation. Each core was sheared once after reaching room temperature (22°C) and an average WBS value was determined for each steak.

Consumer panel

Questionnaires were circulated to university personnel to obtain panelists for the study. Consumer panelists (62) were selected from 180 university personnel families based on family income, ethnic origin, sex and questions dealing with methods of cookery and degree of doneness to which they regularly cooked steaks. Consumers were selected who preferred medium doneness and who grilled steaks on gas, electric or charcoal grills. We also considered demographics to get a representative distribution of age, income levels, ethnic origins, and gender for Lubbock, TX.

Consumer sensory evaluation

Home use tests. Steaks (4) from the four primal cuts (n = 248) were trimmed of all fat to a uniform size and randomly assigned to each consumer for evaluation. Steaks were bagged with identification tags and instructions for cookery, sensory evaluation forms, and a beef steak color guide for doneness (National Livestock and Meat Board, 1978). Instructions explained cooking method (i.e., grilling), evaluation procedures, tenderness rating scales, and phone numbers to contact the researcher with any questions.

A 1 hr training session was held with each individual who received a package of steaks for the family. This training involved explicit instructions on cookery. A display of steaks grilled to differing degrees of doneness was used. Consumers were advised to grill steaks to medium doneness as illustrated in the beef steak color guide.

Each family member older than 15 yr was requested to score an assigned steak on an 8-point scale (8 = extremely tender, 7 = very tender, 6 = moderately tender, 5 = slightly tender, 4 = slightly tough, 3 = moderately tough, 2 = very tough, and 1 = extremely tough) (Cross, 1978). Panelists were asked to rate each steak as "acceptable" or "unacceptable" in tenderness and for overall acceptability. The data collection period was about 2 mo.

Restaurant tests. The same consumers participated in restaurant testing. Each consumer was randomly assigned six loin strip steaks for evaluation. Steaks were thawed at 2°C for about 48 hr, transported to the restaurant on the day of service and stored at 2°C. Vacuum packaging was removed, and a restaurant-type steak pick was labeled with the corresponding five digit assigned steak number and placed in the steak.

Data were collected at Skyviews of Texas Tech, a laboratory restaurant of the Restaurant, Hotel, and Institutional Management (RHIM) pro-

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Table 1—Demographics for consumer panelists evaluating beef tenderness in home and restaurant testing

Characteristic	Number	Percent
Gender		
Female	31	50
Male	31	50
Age in years		
15–18	9	14.5
19–22	6	9.7
23–29	9	14.5
30–39	14	22.6
40 or more	24	38.7
Ethnicity		
American Indian	2	3.2
Asian	3	4.8
Black	5	8.1
Hispanic	9	14.3
White	43	69.4
Education level		
Non-high School Graduate	7	11.3
High School Graduate	15	24.2
Some College	17	27.4
Bachelor's Degree	6	9.7
Post Graduate	17	27.4
Annual household income		
Less than $20,000	11	17.7
$20,000–39,999	14	22.6
$40,000–59,999	26	41.9
$60,000–99,999	11	17.7
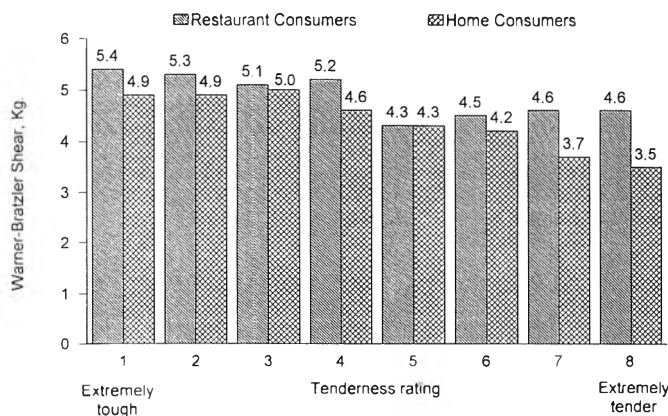
Fig. 1—**Warner-Bratzler shear force values as related to tenderness ratings of steaks by consumers in restaurant and homes.** (Values shown are means, n = 62).
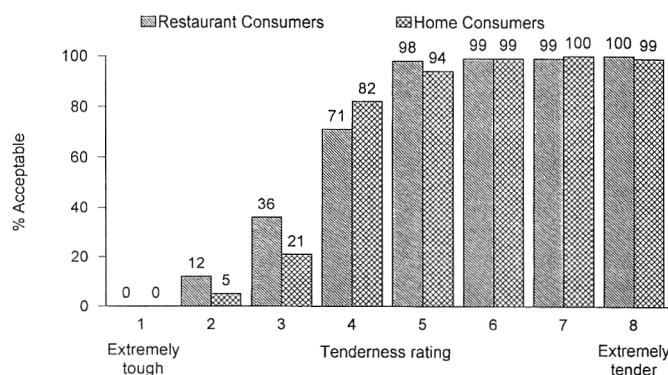
Fig. 2—**Percentage of steaks at each tenderness rating determined to be acceptable in tenderness by consumers in restaurant and homes.** (Values shown are means, n = 62).

gram in the College of Human Sciences, Texas Tech University. Skyviews provides a fine-dining environment with formal table service.

Consumer panelists were asked to attend six, 1.5 hr test meals over a 7-wk period to evaluate 372 steaks. The consumer panel was composed of families, consisting of one to five members, who dined at 5:30 p.m., 6:00 p.m., or 6:30 p.m. Each panelist was assigned the same seat for the six evaluation sessions.

Upon arrival at Skyviews, panelists were seated, and drink orders were taken. Restaurant-type tickets with panelist numbers were given to an expediter in the kitchen. Steaks were pulled according to the panelist numbers on the ticket. At this time the appetizer course, then a salad were served and the grilling process was initiated.

Steaks were placed on the grill, and the numbered pick was placed in a rectangular styrofoam block in the position to correspond with steak on the grill. Steaks were grilled to medium doneness, using a Star-Max Broiler (Star Manufacturing International, Incorporated, Smithville, TN). Internal temperature of steaks was monitored by an Omega DP80 digital thermometer (Omega engineering, Stamford, CT). When the steaks reached 70°C the corresponding numbered pick was inserted back into the steak, and the steak was placed on a dinner plate.

At the completion of the salad course, the entree consisting of steak sample, vegetable medley, roasted new potatoes and freshly baked bread was presented. Wine was offered to the panelists when the entree course was presented. Panelists were instructed not to consume the wine until they had rated the steak. Evaluation notebooks then were distributed to the panelists by the service manager who gave a brief description of evaluation forms, instructed panelists and responded to any questions. Consumers first evaluated overall acceptability. After completing the overall acceptability evaluation, the second form was distributed for evaluation of tenderness, and tenderness acceptability (using the same scales as in the home study). Panelists were given ample time for evaluation of steak samples during the entree course. A dessert course completed the meal.

Statistical analyses. Data were analyzed with the Statistical Analysis System package (SAS Institute, Inc., 1985). Mean WBS values for the paired WBS steaks from both the home and restaurant were calculated for each point on the 8-point tenderness rating scale as were descriptive statistics, including frequency and percentages of consumers rating the steaks "acceptable," for both tenderness acceptability and overall acceptability. For example, of all steaks that received a 2 rating the WBS associated with those steaks were used to calculate the mean for WBS value at the 2 tenderness rating. Correlation coefficients were computed for relationships between tenderness acceptability and overall acceptability.

RESULTS & DISCUSSION

Consumer panel

The demographic characteristics of the 62 consumers were evaluated (Table 1). Age range was 15–52 yr. Over 70% of panelists were white. Other ethnic groups included Hispanic, Black, Asian, American Indian, and African American. Family income ranged from <$10,000 to 99,999, with a median of $40,000–$50,000.

Consumer acceptability scores

Data (Fig. 1) showed as WBS value decreased, tenderness scores increased (indicated more tenderness). This suggested that consumers could detect changes in tenderness similar to those found from the instrumental measurement. In all tenderness ratings except 5, a lower WBS value was associated with a given tenderness rating in the homes than in the restaurant.

The transition in ratings from tough to tender was determined based on data of Fig. 1. Initial transition for WBS values occurred between a 4 (5.2 kg) and 5 (4.3 kg) tenderness rating for consumers in the restaurant setting and between a 3 (5.0 kg) and 4 (4.6 kg) tenderness rating in the homes. Consumers in the restaurant tolerated slightly tougher meat and gave higher tenderness ratings at any given shear value than the same consumers in their homes.

Percentages of steaks were compared (Fig. 2) at each tenderness rating deemed acceptable in tenderness by consumers. The largest increase in tenderness acceptability for all steaks occurred between a 3 and 4 tenderness rating for both home and restaurant consumption. This established the tenderness acceptability level. The beef tenderness acceptability level occurred at 4.3 kg.

In homes and at the restaurant, when steaks were given a minimum of a 5 tenderness rating, at least 94% were rated acceptable for tenderness. When the tenderness rating dropped from 4 to a 3, 60% fewer consumers rated tenderness acceptable in the home. However, in the restaurant only 35% fewer cus-
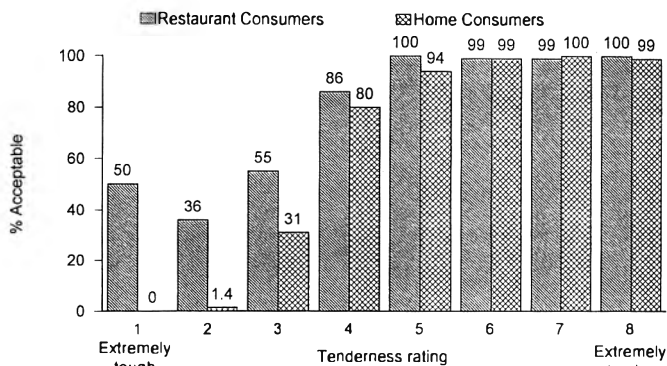
Fig. 3—Percentage of steaks at each tenderness rating determined to be acceptable overall by consumers in the restaurant and homes. (Values shown are means, n = 62).
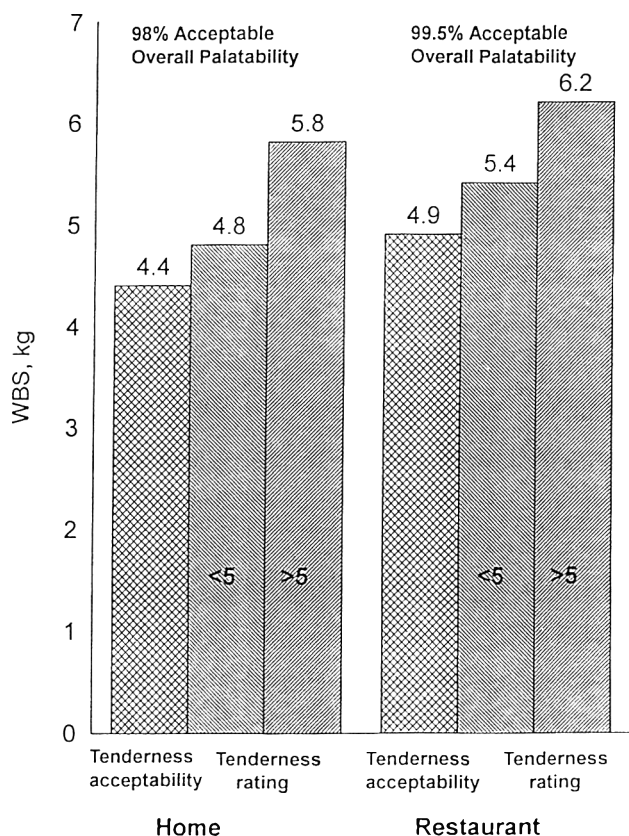
Fig. 4—Tenderness transition levels for steak consumers in homes and the restaurant.

tomers rated the 3-scored steaks acceptable. If the steaks were given a 2 or 3 tenderness rating, a much higher percentage of steaks were rated acceptable in tenderness in the restaurant than in the homes. This appeared to indicate that consumers may be more tolerant of tougher beef in a restaurant than in the home.

Percentages of steaks at each tenderness rating deemed to be "overall acceptable" by consumer panelists were also compared (Fig. 3). Consumers were less critical when rating overall acceptability of steaks as compared to tenderness acceptability. This indicated that tenderness was not the only factor they considered important in determination of acceptability (Fig. 2). In homes and at the restaurant, if steaks were given a minimum of a 4 tenderness rating, 80% or more were rated overall acceptable (the same pattern as for tenderness acceptability). If the score was 3 or lower for tenderness, consumers in the restaurant found a much larger percentage of steaks overall acceptable than they did in their homes. When the tenderness rating was 3, 54% of

the consumers in the restaurant judged the steaks overall acceptable and only 31% of the home consumers considered them overall acceptable. In the restaurant, 36% of the steaks rated very tough were considered overall acceptable while only 1.4% were found acceptable in the home. Similarly, 50% of the steaks that were rated extremely tough were considered overall acceptable in the restaurant and none in the home. No steaks were acceptable when rated extremely tough in the home. Correlation coefficients between tenderness acceptability and overall acceptability in the home were 0.66, in the restaurant 0.73. Thus, tenderness accounted for 44% (R^2) of the variation in overall acceptability in the homes and 53% in the restaurant. The beef industry should focus on beef tenderness as an important trait because it accounts for a considerable degree of variation in acceptability among consumers. However, such results show other attributes, such as juiciness and flavor, may affect overall acceptability of beef steaks. Miller et al. (1995) reported tenderness and flavor to both be important in the consumer acceptability of beef.

Of the total steaks, 81.3% were considered acceptable in tenderness by restaurant consumers and 87.6% acceptable overall. These data show that although some steaks were too tough to be acceptable for tenderness, other attributes such as flavor and juiciness made them acceptable overall.

Relationships between the home and restaurant environment were compared when at least 98% of the steaks were rated overall acceptable (Fig. 4). In the home, when steaks were rated acceptable for tenderness, the average WBS value was 3.5 kg. However, in the restaurant, steaks rated acceptable in tenderness had a WBS average of 4.0 kg. This suggests that less tender steaks were considered acceptable in the restaurant than in the home. The figure supports this finding by separating the tough and tender ratings from the 8-point scale. For the home, when steaks received at least a slightly tender rating (≥ 5), the WBS value was 3.9 and when the steaks received a slightly tough rating (≤ 4) the WBS value was 4.9. In the restaurant, steaks receiving at least a slightly tender rating had a WBS value of 4.5 and for the slightly tough (≤ 4) the WBS value was 5.3. Consumers may also have been influenced by atmosphere, service, convenience and quality of accompanying food when determining overall acceptability of the steaks. They also may have been influenced by receiving a free meal in the restaurant or free steaks for the home portion of the study. Results indicate that there is a level of tenderness (about 4.3 kg) that is unacceptable to steak consumers, and such level can be defined in terms of WBS values.

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Ms received 6/1/94; revised 12/30/94; accepted 5/13/95.

Color Stability and Microbial Growth Relationships in Beef as Affected by Endogenous α-Tocopherol

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————————————— ABSTRACT —————————————

Longissimus muscle from Holstein steers supplemented with vitamin E at 500 or 2000 mg/head/day showed less surface metmyoglobin accumulation than controls during 12 days storage at 4°C. Temperature abuse at 25°C for 24 hr increased metmyoglobin formation; vitamin E supplementation diminished the adverse effect of temperature abuse. No differences (P > 0.05) in bacterial load were observed among the 3 vitamin E treatments during storage. Sensory panelists preferred vitamin E-supplemented beef steaks in visual acceptance. Panelist assessment of discoloration correlated highly with a* value and hue angle. In general, elevated α-tocopherol concentrations in beef steaks did not affect panelist assessment of meat spoilage.

Key Words: beef, color stability, α-tocopherol, microbial

INTRODUCTION

THE APPEARANCE OF MEAT, especially its color, has been used by consumers as an important indicator of meat quality. When oxymyoglobin is oxidized to metmyoglobin in beef, the color changes from acceptable cherry-red to undesirable brown. Technology which extends the color shelf-life of meat would improve the economic return since discoloration-caused conversions, discounts, and discards of products would be reduced (Schaefer et al., 1991). However, extension in color shelf-life of meat products does not necessarily equate with extension of microbiological shelf-life.

Reductants such as sodium ascorbate and sodium erythorbate were reported to maintain color shelf-life in ground beef without affecting bacterial load (Greene et al., 1971). Manu-Tawiah et al. (1991) used a mixture of tetrasodium pyrophosphate, sodium erythorbate and citric acid in combination with modified atmosphere packaging to obtain an extension of color shelf-life in ground beef by 1 to 3 days, and beef steak by 1 day. No differences in growth of mesophiles, psychrotrophs, or enterobacteriaceae were observed between treatments at any storage time. They cautioned that, since bacterial load was unaffected by color maintenance compounds they tested, a potential for "masking" microbial problems could exist with extended color stability. They did not assess spoilage in their study.

Dietary vitamin E supplementation of cattle and pigs improved color stability and shelf-life of beef and pork products (Faustman et al., 1989; Asghar et al., 1991; Lanari et al., 1993). Vitamin E supplementation increased endogenous α-tocopherol concentrations in meat products. However, effects of elevated endogenous tocopherol concentrations on bacterial load have not been adequately studied. Arnold et al. (1992, 1993) found that vitamin E supplementation at levels of 500 and 2000 IU/head/day did not affect total bacterial load on beef from Holstein and beef breed steers during 12 days storage at 4°C. However, Asghar et al. (1991) reported that the bacterial load on pork chops from vitamin E-supplemented pigs was higher than on control pork chops

stored at 4°C for 10 days. They attributed this to a higher drip loss in non-supplemented pork chops which resulted in less surface moisture on the pork. They maintained that this would provide an environment less favorable for bacterial growth, and thus a lower bacterial load on non-supplemented pork chops. Thus, whether meat products with acceptable appearance due to dietary vitamin E supplementation would also have acceptable microbial shelf-life is not clear.

Our objective was to study the effects of vitamin E supplementation on beef color stability, and microbial growth and profile in longissimus lumborum through both instrumental and sensory evaluations.

MATERIALS & METHODS

Animals and diets

Holstein steers (n=44) were used. Three different trials (Trial 1, n=14; Trial 2, n=15; Trial 3, n=15) were performed over a period of 7 mo. All animals in the three trials were given ad libitum access to diets consisting of 90% corn-plus-supplement/10% corn silage (Arnold et al., 1993) at the Univ. of Wisconsin. Cattle weighing 450 kg were assigned randomly to 1 of 3 dl-α-tocopheryl acetate supplementation target levels: 0 mg/head/day (E-0; n=14), 500 mg/head/day (E-500; n=15), 2000 mg/head/day (E-2000; n=15) for 126 days prior to slaughter. For each replicate trial, five animals were randomly assigned to each dietary treatment. However due to an unavoidable error in fabrication and shipping in Trial 1, four rather than five control loins were available. In Trials 2 and 3, there were five different loins available from each of the three treatment groups. Diets were sampled twice per week and samples from two consecutive weeks were composited for vitamin E analysis. The intake of vitamin E was 67 (E-0), 380 (E-500) and 1447 (E-2000) IU/head/day. Steers were slaughtered at Packerland Packing Company (Green Bay, WI) when they were 600 kg. Left strip loins were removed at 24 hr postmortem, vacuum-packed and shipped to the University of Connecticut and stored at 4°C. At 14 days postmortem, the longissimus lumborum (LL) muscle was dissected from the loin and prepared as follows.

Nonabuse and temperature abuse studies

Slices (1 cm thick) were prepared from each longissimus lumborum. Beef cores (12 cm² × 1 cm thick) were removed from the slices, placed on fiberboard trays and overwrapped with oxygen-permeable PVC film (15,500-16,275 cm³/m²/24 hr at 23°C) and stored at 4°C for 12 days under illumination from cool white fluorescent lights (581 lux). One set of beef cores (n=7 per LL) was stored at 4°C (non-abused) for 12 days; the second set of beef cores (n=7 per LL) was stored at 25°C for 24 hr (temperature-abused) and subsequently stored at 4°C for 12 days. The remains of the beef slices were vacuum-packed and stored at −20°C until analyzed for α-tocopherol concentration. Surface metmyoglobin accumulation of non-abused and temperature-abused beef cores was determined on alternate days using a Shimadzu UV-Vis 2100U spectrophotometer equipped with an integrating sphere for diffuse reflectance (Stewart et al., 1965). Duplicate measurements were performed through the film on each core. Immediately after surface metmyoglobin determination, beef cores were analyzed for total aerobic bacterial load. Each beef core was placed in a sterile stomacher bag with 100 mL sterile peptone water (0.1%) and homogenized for 60 sec in a stomacher (400 Mark II, Fisher Scientific). Serial dilutions of the homogenate were applied to duplicate plates of plate count agar (PCA, Difco) and incubated at 25°C for 48 hr (Steinbrugge and Maxcy, 1988). Bacterial counts were expressed on the basis of total surface area of both horizonal faces of the core.
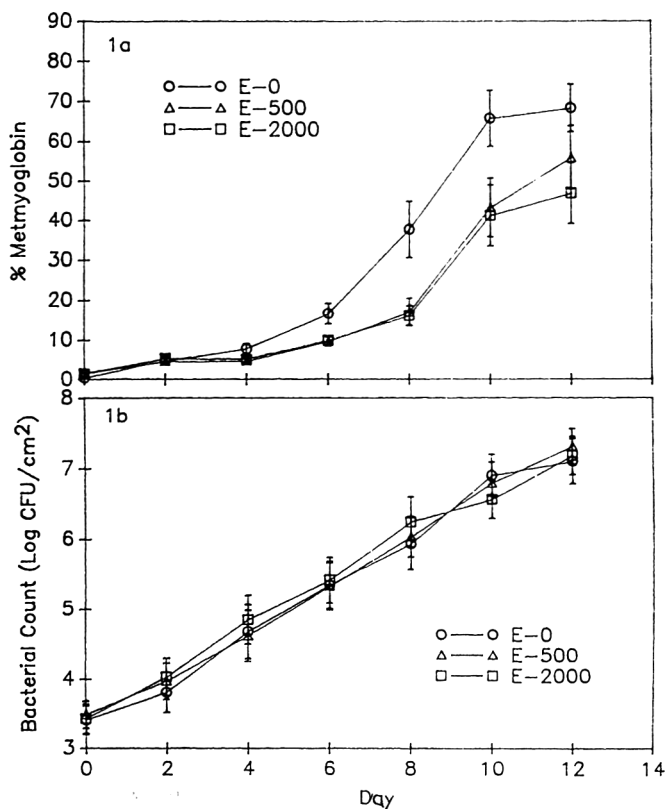
Fig. 1—Surface metmyoglobin (1a) and aerobic plate count (1b) of beef longissimus, lumborum muscle samples obtained from cattle supplemented with vitamin E at levels of 0 (E-0), 500 (E-500), and 2000 (E-2000) mg/head/day and stored at 4°C for 12 days. Standard error bars are indicated.

Bacterial profile

Identification of bacterial isolates from meat cores in the non-abused study was performed in Trials 1 and 2. Five colonies were randomly removed from each countable aerobic plate on day 0, 4 and 8 for identification according to Manu-Tawiah et al. (1991). Selected colonies were spread on trypticase soy agar (TSA) and plates were incubated at 25°C for 24 hr to assess purity. Isolates from TSA were characterized initially according to a Gram stain reaction. Gram-negative isolates were separated initially based on FeS formation in Triple Sugar Iron Agar (TSI; Difco) and presence of urease. Isolates with the same TSI reaction, urea reaction and cell morphology were grouped together and one isolate/group was identified using API 20E test strips (BioMerieux Vitek Inc, Hazelwood, MO). Gram-positive isolates were identified following the scheme of Harrison et al. (1981).

Sensory studies

Sensory evaluation experiments were conducted in a double-blind manner in individual booths with a semi-trained panel consisting of university staff (n=11 members, trial 1; n= 10 members, trial 2; n=8 members, trial 3). It was desired to have panelists evaluate discoloration and spoilage status of beef steaks which had been exposed to display conditions for a wide range of exposure times. In order to avoid scheduling conflicts and the confounding effect of day of evaluation, strip loin steaks (2 cm) were removed from each loin at 14, 11, 8 and 4 days, and 2 hr prior to evaluation. For each time point at which steaks were sliced in advance of the sensory evaluation date, primals were again vacuum-packed. Prior to removal of a steak from the LL, a thin slice (1 cm) was removed to provide a fresh cut surface. Beef steaks were placed on fiberboard trays and overwrapped as described. Panelists were requested to evaluate each steak for presence and extent of discoloration, and acceptability of appearance for purchase. In addition, panelists assessed spoilage status in terms of olfactory acceptability and indicated whether or not they would consume the steak. Following completion of the panel evaluation, each steak was analyzed for total bacterial load and color status. One core (12 cm² × 2 cm thick) was removed from the geometric center of each steak and analyzed for bacterial load as described. For instrumental color analysis, four measurements were made around the core periphery using a Minolta Chro-

mameter CR 200; a*, b* and L* values were recorded and hue angle calculated as: hue angle=tan⁻¹(b*/a*)*360°/2π.

α-Tocopherol analysis

Alpha-tocopheryl acetate concentrations in diet samples and α-tocopherol concentrations in LL were determined according to Arnold et al. (1993) using normal-phase, isocratic high-performance liquid chromatography with fluorescence detection. Determinations were made on duplicate 1-g aliquots of each samples.

Statistical analysis

Results from the three trials were not different and thus data were pooled together for subsequent analysis. Data were analyzed by the General Linear Models procedure of SAS Institute, Inc. (1985) with a split-split plot design to account for repeated measurements. Animals were considered as the main plot, animal × vitamin E treatment as sub-plot, and animal × vitamin E treatment × day as sub-sub-plot. Bacterial load data were subjected to a log transformation prior to statistical analysis. Differences between vitamin E treatment means were detected by the LSD multiple range test. Regression analyses were performed and the significance of correlation coefficients was determined by the t statistic where, $t=(r)(n-2)^{1/2}/(1-r^2)^{1/2}$, with n-2 degrees of freedom.

RESULTS

THE ACCUMULATION OF METMYOGLOBIN on nonabused beef cores during storage at 4°C for 12 days was assessed (Fig. 1a). No differences (P > 0.05) in surface metmyoglobin accumulation were observed between treatments during the first 4 days of storage. When stored for more than 6 days, E-0 beef cores showed greater surface metmyoglobin accumulation than E-500 and E-2000 treatments (P < 0.05). Surface metmyoglobin accumulation on E-500 and E-2000 beef cores did not differ over the 12-day storage (P > 0.05; Fig. 1a). No difference in total bacterial load was found among the three treatments for any storage time (P > 0.05; Fig. 1b). Presumptive identification of bacterial genera in the non-abuse study indicated no treatment effect for any mesophilic genus present on beef cores stored at 4°C for 0, 4 or 8 days (Table 1). The Pseudomonas and Coryneform groups were identified as predominant mesophilic microflora; the proportion of Pseudomonas isolates appeared to increase during storage (Table 1).

Temperature abuse of beef cores increased metmyoglobin formation and total bacterial load on beef core surfaces (Fig. 2a and 2b) when compared with nonabused counterparts (Fig. 1a and 1b; P < 0.05). During subsequent storage at 4°C for 6 days, surface metmyoglobin accumulation in temperature-abused samples was higher in E-0 than E-500 and E-2000 (P < 0.05). Following temperature abuse, E-0, E-500 and E-2000 beef cores demonstrated no difference in bacterial growth over 12 days storage at 4°C (P > 0.05; Fig. 2b).

Vitamin E supplementation at levels of 500 and 2000 mg/head/day increased endogenous tocopherol concentrations of LL muscle over that of E-0 by 2.6- and 5.5-fold, respectively (Table 2). The relationship between endogenous tocopherol concentrations and metmyoglobin accumulation on non-abused beef cores at day 8 of 4°C storage was compared (Fig. 3). Higher endogenous tocopherol concentrations in beef correlated with lower metmyoglobin level; a threshold concentration of 4 μg α-tocopherol/g muscle appeared necessary to maximally retard metmyoglobin formation.

Sensory evaluation responses were compared (Table 3). On day 0, steaks from E-0, E-500 and E-2000 treatments were considered of normal color and had acceptable appearance; no difference was found among the three treatments (P > 0.05). When steaks were stored at 4°C for 4 days, more panelists considered E-0 steaks discolored than E-500 and E-2000 (P < 0.05), however, no treatment effect was evident for appearance acceptance and purchasing preference (P > 0.05). At 8 to 14 days storage, essentially all steaks were considered discolored to some extent by panelists. However, more panelists accepted the appearance of E-500 and

Table 1—Genera of mesophilic isolates (n = 45, E-0; n = 50, E-500, and n = 50, E-2000) obtained from beef cores stored at 4°C for 0, 4 and 8 days

| Genus | No. of isolates | | | | | | | | |
| | Day 0 | | | Day 4 | | | Day 8 | | |
	E-0	E-500	E-2000	E-0	E-500	E-2000	E-0	E-500	E-2000
Gram-negative									
Pseudomonas	17	22	22	23	22	27	26	30	31
Acinetobacter	1	4	3	4	4	2	3	0	0
Serratia	2	0	0	1	0	1	1	0	0
Hafnia	1	0	0	0	0	0	0	0	0
Klebsiella	0	1	0	0	0	0	0	0	0
Gram-positive									
Coryneform	15	14	20	15	20	15	13	14	16
Micrococcus	7	3	2	1	1	2	0	0	0
Lactobacillus	0	4	0	1	1	0	2	2	1
Lost (no growth upon transfer)	2	2	3	0	2	3	0	4	2
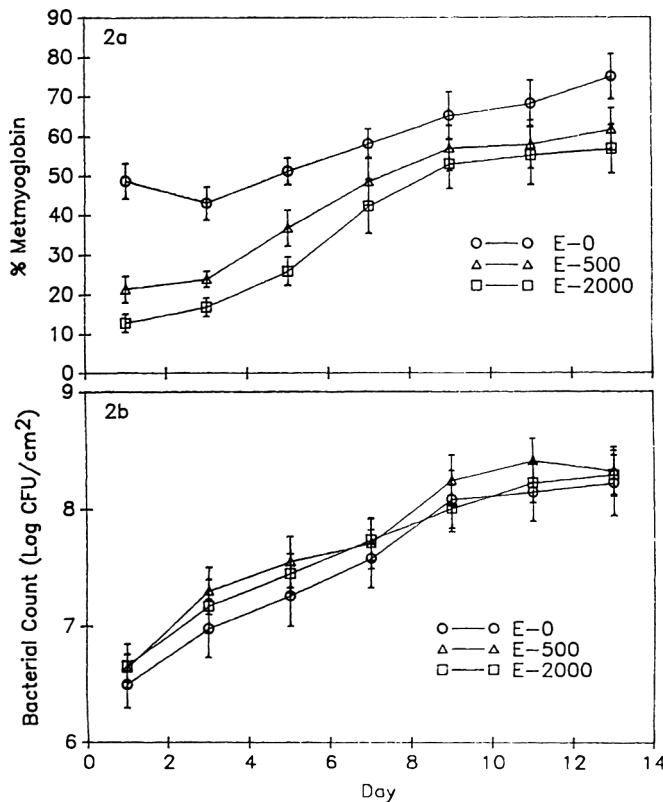
Fig. 2—Surface metmyoglobin (2a) and aerobic plate count (2b) of beef longissimus lumborum muscle samples obtained from cattle supplemented with vitamin E at levels of 0 (E-0), 500 (E-500) and 2000 (E-2000) mg/head/day. Beef loin samples were temperature-abused at 25°C for 24 hr and subsequently stored at 4°C for 12 days. Standard error bars are indicated.

Table 2—Alpha-tocopherol concentrations in longissimus lumborum muscle from cattle supplemented with vitamin E at levels of 0 (E-0), 500 (E-500) and 2000 (E-2000) mg/head/day for 126 days

| | Treatments | | |
	E-0	E-500	E-2000
α-Tocopherol concentrations (µg/g muscle)	1.11 ± 0.05[a]	2.90 ± 0.10[b]	6.16 ± 0.16[c]

[a-c] Means with different superscripts are different (P < 0.05).

E-2000 steaks and preferred purchasing them when compared to E-0 steaks (P < 0.05).

The panelists' estimates of steak surface discoloration were compared (Fig. 4a). Over a 14-day storage, surface discoloration followed the order E-0 > E-500 > E-2000 (P < 0.05). The $a*$ value readings (Fig. 4b) and hue angle values (Fig. 4c) of loin
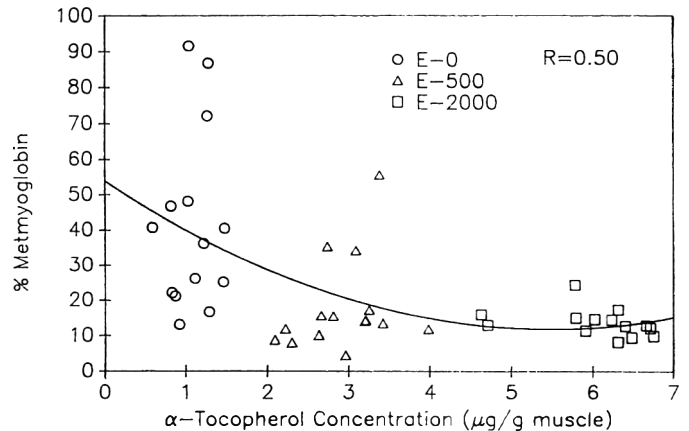
Fig. 3—Relationship between α-tocopherol concentration and percent metmyoglobin on day 8 of display at 4°C for beef longissimus lumborum muscle samples from cattle supplemented with vitamin E at levels of 0 (E-0), 500 and 2000 (E-2000) mg/head/day. A second order regression is indicated.

steaks supported this sensory result in that a decrease in redness and increase in hue angle followed the order E-0 > E-500 > E-2000 (P < 0.05). The instrumental measurement of hue angle value and sensory panelist assessment of discoloration demonstrated similar responses (Fig. 4a and 4c). Correlations between estimated surface discoloration and $a*$ value (R=0.84; Fig. 5a) and hue angle value (R=0.88; Fig. 5b) were significant (P < 0.05). There were no treatment differences in $L*$ or $b*$ values (data not shown).

Sensory evaluation results indicated that olfactory quality of beef steaks was similar for E-0, E-500 and E-2000 steaks for days 0, 4, 8 and 14 of storage (Table 3). For day 11 steaks, fewer panelists detected spoilage in E-2000 than in E-0 steaks (Table 3). There was no difference in olfactory quality between E-0 and E-500, or E-500 and E-2000 steaks (P > 0.05). Similar to results for beef cores, total aerobic bacterial load on beef steaks was not affected by vitamin E treatment (P > 0.05; Fig. 4d).

Panelists also judged the overall quality of beef steaks by indicating their consumption preference based on combined visual and olfactory assessment (Table 3). During the early storage period, panelists did not discriminate between vitamin E treatments for consumption preference. When steaks were stored 8 days or longer at 4°C, more panelists indicated a preference for consumption of E-500 and E-2000 steaks over E-0 steaks (P < 0.05). In no instance did panelists indicate that they would choose to consume a beef steak which had been identified as spoiled (data not shown). On day 4 of storage, 3 to 6% of panelists would not consume beef steaks since they perceived them as spoiled, although they indicated a willingness to purchase based on appearance. However, there was no main treatment effect in this regard. On day 8 of storage, 4 to 6% of panelists were willing to

Table 3—Sensory evaluation of loin steaks from cattle supplemented with vitamin E at levels of 0 (E-0), 500 (E-500) and 2000 (E-2000) mg/head/day

| Category | Trt | Days stored at 4°C | | | | |
| | | 0 | 4 | 8 | 11 | 14 |
		Percent of panelists responding "yes"				
Visual qualities						
Is the steak discolored?	E-0	0 ± 0[a]	75.0 ± 6.1[b]	100 ± 0[d]	100 ± 0[d]	100 ± 0[d]
	E-500	1.3 ± 0.9[a]	43.2 ± 7.6[c]	97.4 ± 1.4[d]	99.4 ± 0.6[d]	100 ± 0[d]
	E-2000	2.2 ± 1.1[a]	48.4 ± 6.7[c]	92.8 ± 3.0[d]	100 ± 0[d]	100 ± 0[d]
Is the steak's appearance acceptable?	E-0	100 ± 0[a]	95.7 ± 1.4[a]	40.2 ± 8.8[c]	6.61 ± 3.2[e]	5.0 ± 2.7[e]
	E-500	100 ± 0[a]	98.7 ± 1.3[a]	75.2 ± 5.5[b]	36.6 ± 9.0[c]	19.5 ± 7.4[d]
	E-2000	100 ± 0[a]	100 ± 0[a]	88.8 ± 3.4[ab]	40.4 ± 6.0[c]	24.7 ± 8.3[d]
Would you buy the steak?	E-0	100 ± 0[a]	95.7 ± 1.4[a]	37.7 ± 7.9[c]	7.3 ± 3.2[e]	5.0 ± 2.7[e]
	E-500	100 ± 0[a]	98.7 ± 1.3[a]	74.9 ± 5.4[b]	38.6 ± 8.6[c]	18.9 ± 7.3[d]
	E-2000	100 ± 0[a]	100 ± 0[a]	88.2 ± 3.5[ab]	41.1 ± 6.0[c]	25.2 ± 7.9[d]
Olfactory quality						
Is the steak spoiled?	E-0	0 ± 0[a]	7.1 ± 1.2[b]	15.2 ± 2.3[c]	25.7 ± 3.9[d]	73.7 ± 5.4[e]
	E-500	0 ± 0[a]	5.1 ± 1.5[b]	12.4 ± 4.6[c]	22.5 ± 4.1[cd]	62.1 ± 8.6[e]
	E-2000	0 ± 0[a]	3.2 ± 1.2[b]	11.4 ± 2.2[c]	15.4 ± 2.5[c]	61.4 ± 8.3[e]
Overall quality assessment						
Would you consume the steak?	E-0	100 ± 0[a]	90.9 ± 1.8[a]	55.6 ± 5.0[c]	31.5 ± 3.2[d]	13.1 ± 2.7[e]
	E-500	100 ± 0[a]	94.9 ± 1.6[a]	74.8 ± 3.9[b]	51.9 ± 5.5[c]	25.1 ± 6.6[d]
	E-2000	100 ± 0[a]	96.9 ± 1.2[a]	77.1 ± 2.4[b]	50.8 ± 5.2[c]	25.8 ± 6.4[d]
Purchase but note spoilage and do not consume it[f]	E-0	0 ± 0[a]	5.5 ± 1.3[b]	0.7 ± 0.7[a]	0 ± 0[a]	0 ± 0[a]
	E-500	0 ± 0[a]	3.9 ± 1.2[b]	5.3 ± 1.6[b]	0.8 ± 0.8[a]	0 ± 0[a]
	E-2000	0 ± 0[a]	3.2 ± 1.2[b]	4.6 ± 1.8[b]	0.6 ± 0.6[a]	0 ± 0[a]

[a-e] Within the same question, treatment means (± SE) with different superscripts are different (P < 0.05).
[f] Percentage of panelists (calculated from responses) who would purchase the steak but note spoilage and not consume it.
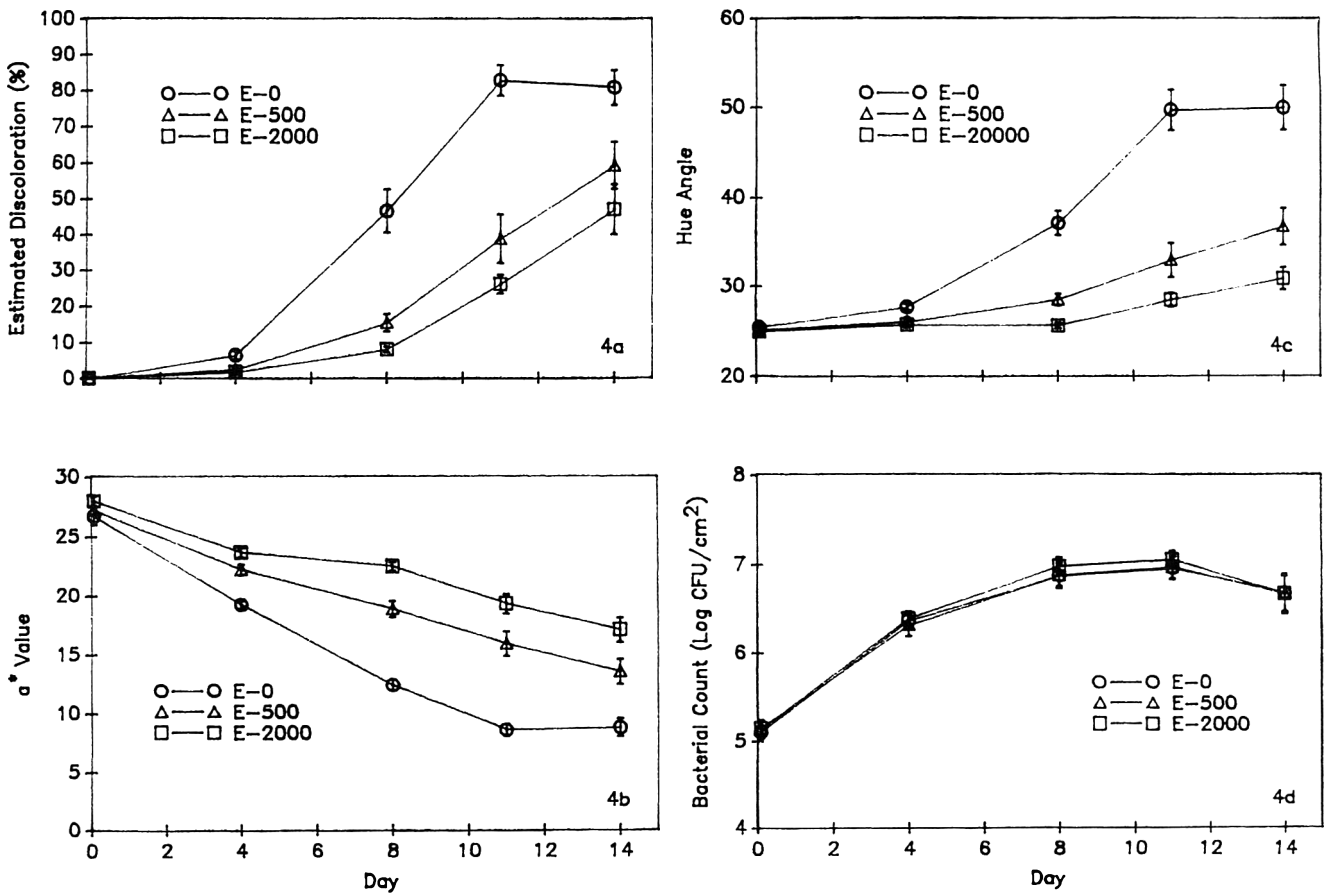
Fig. 4—Sensory analysis of surface discoloration (%)(4a); objective analysis of a* value (4b) and hue angle (4c); and total bacterial load (4d); of beef loin steaks from cattle supplemented with vitamin E at levels of 0 (E-0), 500 (E-500) and 2000 (E-2000) mg/head/day. Beef steaks were stored at 4°C for 14 days. Standard error bars are indicated.

purchase E-500 and E-2000 beef steaks but would not consume them due to spoilage; only 0.7% of panelists responded in this manner for E-0 steaks. However, it is important to note that pan-

elist response for E-500 and E-2000 steaks on day 8 was not different from that of E-0 steaks on day 4 (P > 0.05). On days 11 and 14 storage, < 1% of panelists indicated that purchased
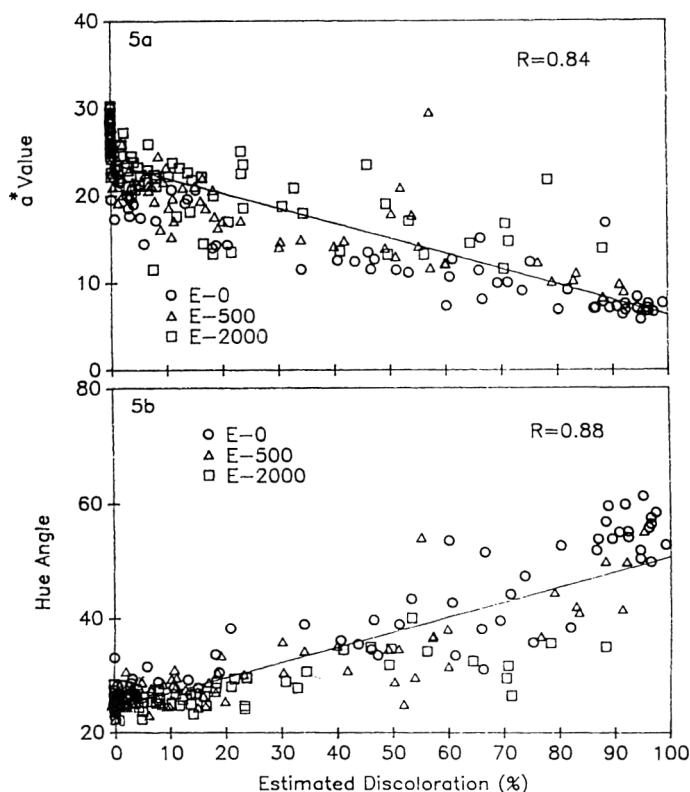
Fig. 5—Relationship between estimated discoloration (%) and a* value (5a); and hue angle (5b) of beef loin steaks from cattle supplemented with vitamin E at 0 (E-0), 500 (E-500) and 2000 (E-2000) mg/head/day. Beef steaks were stored at 4°C for 14 days. Standard error bars are indicated.

steaks would not be consumed due to spoilage regardless of treatments.

DISCUSSION

DIETARY VITAMIN E SUPPLEMENTATION was effective in decreasing metmyoglobin formation in LL muscle during 4°C storage and under conditions of temperature abuse (Fig. 1a and 2a). Mitsumoto et al. (1991) reported a negative correlation between α-tocopherol concentrations and metmyoglobin formation in Holstein and crossbred beef at day 16 of 4°C storage. They suggested that 3.5 μg/g muscle was needed to retard metmyoglobin formation. Arnold et al. (1992) also reported a correlation between α-tocopherol concentration and metmyoglobin formation in loin and sirloin. Metmyoglobin formation approached a minimum of 23% at 3.3 μg/g longissimus muscle stored at 4°C for 12 days and 26% at 3.8 μg/g gluteus medius muscle stored at 4°C for 8 days. A strong correlation between α-tocopherol concentration and metmyoglobin accumulation was also observed in our study (Fig. 3). However, $\approx$ 4 μg α-tocopherol/g beef appeared necessary for retarding metmyoglobin formation; a value slightly higher than those reported by Mitsumoto et al. (1991) and Arnold et al. (1992).

Although the α-tocopherol concentration was 2.2 fold higher in E-2000 than in E-500 beef (Table 2), metmyoglobin accumulation did not differ among the vitamin E treatments (P < 0.05; Fig. 1a and 2a). Arnold et al. (1993) found no differences in metmyoglobin accumulation during retail display of longissimus muscle from cattle which received 380 and 1446 IU/day for the last 100 days of a 252-day study. Although spectrophotometric determination of metmyoglobin accumulation in beef cores revealed no difference in color preserving effect between E-500 and E-2000, panelists in our double-blind study discriminated between the treatments based on estimated discoloration (Fig. 4a). The perception of the panelists was consistent with the discrimination

among treatment effects revealed by colorimetric assessment of a* value and hue angle. Hue angle appeared especially sensitive as a measure of beef color stability.

Sensory evaluation indicated that estimated discoloration (%) of beef steaks strongly influenced panelist response regarding appearance acceptability and purchase preference (Fig. 4a and Table 3). When beef steaks were perceived to contain < 15% discoloration, more than 75% of panelists accepted the appearance. However, once panelists perceived steaks to be > 20% discolored, > 50% of panelists rejected the steaks. This result was similar to that of Hood and Riordan (1973) who reported that shopper discrimination against discolored meat increased with increase in metmyoglobin content. The ratio of sales of discolored beef to bright red beef was $\approx$ 1:2 when 20% metmyoglobin was present in the discolored batch. Beef steaks from vitamin E supplemented cattle were perceived to be less discolored than those from non-supplemented animals after 4 days storage at 4°C (Fig. 4a), and more favorable in appearance by panelists (Table 3). These results further confirmed previous studies that dietary vitamin E supplementation extends color shelf life of fresh beef (Faustman et al., 1989; Mitsumoto et al., 1991; Arnold et al., 1992).

Endogenous α-tocopherol concentrations of LL muscle were increased by vitamin E supplementation (Table 2). Elevated α-tocopherol concentrations in vitamin E-supplemented beef had no influence on bacterial load during 4°C storage (Fig. 1b and Fig. 4d) or during temperature abuse (Fig. 2b). These results were consistent with findings by Arnold et al. (1992, 1993) who showed no effect of dietary vitamin E on bacterial load in beef during the display period, but differ from results of Asghar et al. (1991) who reported higher bacterial load in vitamin E-supplemented than in control pork chops. In addition, the profile of mesophilic microflora on beef in the non-abuse study was unaffected by dietary vitamin E supplementation (Table 1). Manu-Tawiah et al. (1991) reported that use of tetrasodium pyrophosphate, sodium erythorbate and citric acid to extend beef color shelf-life had no effect on presence of certain mesophilic bacterial genera.

Elevated temperature or extended storage accelerated oxymyoglobin oxidation and bacterial growth in beef (Fig. 1 and 2). Bala et al. (1977) reported a concomitant decrease in oxymyoglobin content with increased bacterial load and suggested that bacteria may cause oxymyoglobin oxidation. O'Keeffe and Hood (1982) hypothesized that increased oxygen consumption, which might be caused by bacterial growth, may lower partial oxygen pressure sufficiently to enhance oxymyoglobin oxidation. However, Bevilacqua and Zaritzky (1986) provided data which failed to support a bacterial:pO$_2$ mechanism for accelerating meat discoloration. In our results, no difference in bacterial load resulted from vitamin E supplementation (Fig. 1b and 2b), yet there was less discoloration in E-500 and E-2000 steaks. In view of these results, significance of the relationship between bacteria and meat discoloration must be reconsidered. Thus, for a given level of discoloration, bacterial load was greater in beef from vitamin E-supplemented cattle than from controls. Results from Manu-Tawiah et al. (1991) also showed that bacterial loads were greater in color-stabilized beef than controls for a given level of discoloration. In the temperature abuse study, bacterial load and metmyoglobin formation were substantially higher following 24 hr at 25°C than in the non-abused samples (P < 0.05). However, there was a clear vitamin E treatment difference for pigment oxidation, but not bacterial load. These results did not support a clear-cut relationship between discoloration and bacterial load.

Spoilage of beef occurs when the bacterial load reaches ca. 10^7 CFU/cm^2 (Kraft, 1986). In our study, this level occurred on beef steak by day 8 of storage at 4°C (Fig. 4d). Longissimus muscle has a color shelf life of ca. 6 days (O'Keeffe and Hood, 1982). Arnold et al. (1993) reported that vitamin E supplementation could extend the color shelf-life of longissimus muscle by 2 to 5 days and thus provide a substantial economic advantage. Dietary vitamin E had no effect on bacterial load, and spoilage assessment

of beef steaks was generally not affected by treatment. The reason that fewer panelists detected spoilage on E-2000 steaks than E-0 steaks for day 11 is unknown. Panelists may associate cherry-red color with less bacterial load, which influenced their perception for spoilage, but this did not hold for day 14.

A potential problem with the use of vitamin E for extending color shelf-life is that consumers may purchase beef based on acceptable appearance, but then subsequently determine that it is spoiled. There was no difference between control and E-treated beef steaks stored at 4°C for 0, 4, 11 and 14 days (P > 0.05; Table 3). When beef steaks were stored at 4°C for 8 days, more panelists would have purchased spoiled steaks with good appearance from the vitamin E treatments than the control treatment. The relative number of panelists actually doing this was < 10% (Table 3) and no different than the percentage of panelists responding similarly for E-0 steaks on day 4 (P > 0.05). Meat cuts with lower color stability than LL muscle, such as psoas major and gluteus medius, may respond to vitamin E supplementation differently; this could also impact panelist response (Chan et al., 1995).

CONCLUSION

DIETARY VITAMIN E SUPPLEMENTATION of Holstein steers at levels of 380 (E-500) and 1447 (E-2000) ng/head/day for 126 days decreased surface metmyoglobin formation of beef under conditions of normal refrigerated storage at 4°C and temperature abuse. Vitamin E supplementation also increased color stability of beef steaks and visual acceptance by panelists. No significant effect was observed for vitamin E supplementation on bacterial load of beef. Panelist assessment of meat spoilage was not affected by dietary vitamin E supplementation.

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Survival of *Listeria monocytogenes, Yersinia enterocolitica* and *Escherichia coli* O157:H7 and Quality Changes After Irradiation of Beef Steaks and Ground Beef

AN-HUNG FU, J. G. SEBRANEK, and E. A. MURANO

——————————— ABSTRACT ———————————

Beef steaks and ground beef were inoculated with *Listeria monocytogenes, Yersinia enterocolitica,* or *Escherichia coli* O157:H7. Samples were packaged in air or under vacuum and irradiated at low (0.60 to 0.80 kGy) or medium (1.5 to 2.0 kGy) doses, with each dose delivered at either a low (2.8 M/min conveyor speed) or high (6.9 M/min) dose rate. Medium-dose irradiation accompanied by 7°C storage resulted in no *Y. enterocolitica* or *E. coli* O157:H7 survivors being detected. There was no effect on survival of the pathogens by dose rate or storage atmosphere. No difference (P>0.05) was observed in meat pH or color, but TBA values increased after 7 days storage.

Key Words: beef, pathogens, microbials, irradiation

INTRODUCTION

IN JANUARY 1993, an outbreak of foodborne illness caused by consumption of hamburger contaminated with *Escherichia coli* O157:H7 (Mermelstein, 1993) showed how pathogens can contaminate meat products, survive, grow, and cause disease when proper processing has not been applied. Good manufacturing practices (GMP) and hazard analysis and critical control point (HACCP) systems have been emphasized in slaughtering and processing plants (Tompkin, 1983; Karr et al., 1994), but sporadic contamination is still possible.

There have been several reports on contamination levels of *Listeria monocytogenes* (Johnson et al., 1990), *Yersinia enterocolitica* (Hanna et al.. 1976; Myers et al., 1982), and *Escherichia coli* O157:H7 (Doyle and Schoeni, 1987) in beef. Because these organisms can survive refrigeration temperatures, the use of low-temperature storage alone cannot be relied upon to keep meat safe from them (Palumbo, 1986). Irradiation provides an alternative that can decrease the microbial load of foods and may also eliminate specific pathogens on meat and meat products without changing their nutritive and sensory qualities (Niemand et al., 1981; Thayer et al., 1986; Skala et al., 1987; Thayer, 1993). The Food and Drug Administration (FDA) has approved use of irradiation to reduce bacterial contamination of raw poultry (FDA, 1990), but no approval has been granted for beef. Research is required to demonstrate the benefits of irradiation in preventing outbreaks of foodborne illness from beef or beef products. Research (Clavero et al., 1994) has indicated that pathogens such as *E. coli* C157:H7, salmonellae and *Campylobacter jejuni* are sensitive to gamma irradiation. A dose of 2.5 kGy was suggested as sufficient to kill large numbers of the pathogens in ground beef.

The effect of irradiation dose rate on destruction of microorganisms is controversial. It has been hypothesized that, at a high dose rate, recombination of radicals formed during irradiation takes precedence over reaction of radicals with food components, as occurs at a low dose rate (Hayashi, 1991). This may increase chances for microbial survival, because of more rapid depletion of toxic radicals in the environment. Also, it has been

postulated that high-dose rate irradiation may result in creation of an anoxic environment, resulting in fewer oxygen radicals. Some reports have shown no difference in effects on microorganisms between high dose rates and low dose rates, as demonstrated by comparing gamma irradiation (low dose rate) and electron irradiation (high dose rate) (Hayashi, 1991). Such differences, however, could be due to inherent differences in radiation source, independent of dose rate. It would be valuable to determine whether there is an effect due to dose rate using a given source of irradiation.

Vacuum packaging has been used for primal and subprimal cuts to inhibit growth of spoilage microorganisms in fresh meat. Refrigeration is also important with temperatures between 1°C and 5°C important to delaying microbial spoilage (Gill and Newton, 1978). Most spoilage microorganisms in meat are gram negative, with Pseudomonas predominant when storage is aerobic at refrigeration temperatures. Vacuum packaging will shift the microflora from gram-negative to gram-positive cells, thus delaying meat spoilage. Early studies have shown that lactic acid bacteria predominate in refrigerated vacuum-packaged pork (Lee et al., 1985) and beef (Beebe et al., 1976; Christopher et al., 1979). However, defects such as off-flavors, off-odors, and undesirable colors after 8-11 wks refrigerated storage have been reported (Pierson et al., 1970; Johnson, 1974). Vacuum packaging is often regarded as a modified atmosphere in the sense that elevated levels (10–20%) of CO_2 are produced within the package by respiration of microorganisms and meat. Therefore, safety concerns which have been raised for meat packaged under modified atmospheres need to be considered in vacuum packaging (Hintlian and Hotchkiss, 1986) as well. There has been some concern that modified-atmosphere packaging in combination with low-dose irradiation (<10 kGy) may inhibit some spoilage microorganisms and result in a favored, less-competitive environment for surviving pathogens to proliferate (Lambert et al., 1991).

The objectives of this study were to: evaluate the effects of low and medium doses of irradiation on survival of *Listeria monocytogenes, Yersinia enterocolitica,* and *Escherichia coli* O157:H7 in beef loins, steaks, and ground beef; to determine the effect of dose rate on survival of these pathogens; and to monitor quality changes of pH, lipid oxidation, color, and odor after irradiation at various doses and dose rates.

MATERIALS & METHODS

Sample preparation

Ten vacuum-packed beef rib eye rolls were obtained directly from the processing line of a commercial source and cut into 2.5-cm thick steaks at the Iowa State University Meat Laboratory. Fresh ground beef (80% lean) was obtained from a local supermarket in 2.75 kg lots and separated into 25-g portions to be used as experimental units.

External inoculation and packaging

Steaks and ground beef samples were separated into four groups for inoculation with *L. monocytogenes, Y. enterocolitica,* or *E. coli* O157: H7. Uninoculated samples of each were used as controls. *Listeria monocytogenes* Scott A was grown in trypticase soy broth containing 0.6%

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yeast extract (TSB+YE) at 35°C for 24 hr to the stationary phase. The culture was then transferred to fresh broth and incubated for 6 hr to reach log phase (as determined by previous growth curve experiments). A 2-mL inoculum was transferred to a 10-mL TSB+YE broth and diluted in 1200-mL of 0.1% peptone water to a final concentration of 7 log cells/mL. Using the same procedure, *Y. enterocolitica* was grown in Brain Heart Infusion broth (BHI) at 25°C, and approximately 7 log cells/mL was obtained; *E. coli* O157:H7 was grown in trypticase soy broth (TSB) at 35°C for 12 hr, transferred to fresh broth and incubated for 4 hr to reach log phase. After dilution, the final concentration of *E. coli* O157:H7 was 7 log cells/mL.

Steaks were dipped in the appropriate inoculum solution for 10 min and drained on sterilized racks for 2 min. Inoculations resulted in about 5 log cells/g. Inoculated and uninoculated treatment groups were individually divided into two subgroups, which were assigned to vacuum or in-air packaging. Steaks were placed in high-barrier pouches with O₂ permeability of <2.5 cm³/645 cm²/24 hr at 23°C and 0% RH and water vapor transmission of <1.0 g/645 cm²/24 hr at 38°C and 90% RH (Curlon™ 863 Saran, Curwood Inc. New London, WI). Vacuum packaging was completed by using a model A300 CVP machine (CVP System, Inc., Downers Grove, IL). Vacuum-packed steaks were used to simulate wholesale cuts except for reduced size. Another subgroup of samples was placed in the same type of pouches sealed without vacuum, to simulate steaks packaged with exposure to air. After packaging, all steaks were placed in cardboard boxes and stored at 2–4°C for 12 hr before being irradiated.

Each ground beef unit was inoculated with 1 mL of *Listeria monocytogenes*, *Yersinia enterocolitica*, or *E. coli* O157:H7 from prepared inoculum (1:100 dilution) resulting in about five log cells/g. A high inoculum was used to make enumeration of survivors easier. Once again, uninoculated samples were used as a control set. Samples were wrapped in Saran Wrap™ (Dow Chemical Co., Indianapolis, IN) and were stored at 2–4°C for 12 hr before being irradiated.

Irradiation

Steaks were irradiated at the Linear Accelerator Facility at ISU. The temperature of the product just prior to and immediately after irradiation was monitored by a thermocouple datalogger (Li-con, Stanford, CT) and did not exceed 10°C. Each vacuum or air subgroup was further divided into five groups and assigned to different irradiation-processing treatments. Low or high dose rates were produced by changing the power level (1 or 3 kW) and conveyor speed (cm/min, for short or long exposure time). Doses used were 0.60 kGy and 1.5 kGy, each at low (3.8 or 1.8 M/min conveyor speed) and high (10.2 or 3.9 M/min) dose rate, with nonirradiated samples used as controls. Actual absorbed doses were measured by placing alanine pellets on both sides of a steak with each pellet enclosed within a high-barrier paper pouch to prevent moisture from the product from reaching the pellets. An Electron Paramagnetic Resonance (EPR) instrument was used to determine the absorbed dose (Bruker Instruments, Inc., Billerica, MA). The instrument was calibrated with standard dosimeters provided by the National Institute of Standards and Technology (NIST). Reported doses were averages from doses observed at the top surface and bottom surface of the samples. Variation between minimum and maximum doses were ± 0.1 for low doses and ± 0.2 for medium doses. The same protocol was used for treating ground beef, except that doses were 0.80 kGy and 2.0 kGy, with either a low (3.6 and 1.7 M/min) or high (7.7 and 3/6 M/min) dose rate.

All combinations of treatments for steaks and ground beef were analyzed immediately after irradiation (day 0). Samples were also stored at 7°C for 7 days to simulate consumer storage, followed by temperature abuse at 25°C for 2 days to simulate mishandling. Preliminary studies have shown that storage of fresh meats at 2–4°C (simulated wholesale/retail storage) increased total plate counts (TPC) by only 1.0 log CFU/g; therefore, 7°C was used to represent the temperature of a household refrigerator.

Physical and chemical analyses

Measurements of pH, lipid oxidation (TBA), and color were conducted on uninoculated samples except for products for which the TPC showed obvious spoilage (>10⁷ CFU/g) or when obvious off-odor developed. Sample pH was measured by using a slurry of 10g of meat with 100 mL distilled water, a pH meter (Fisher Accumet Model 925, Fisher Scientific, Pittsburgh, PA), and a sealed combination electrode with silver/silver chloride reference (Omega Engineering, Inc., Stamford, CT). Measurement of thiobarbituric acid (TBA) values, an index of lipid oxidation, was performed by the method of Tarladgis et al. (1960). Du-

plicate readings for each sample were recorded for pH and TBA tests. Color (L, a, and b) measurements were made with a HunterLab Labscan spectrophotometer (Hunter Associated Laboratories Inc., Reston, VA). An average value was obtained from three random surface locations on each sample.

Microbiological analyses

Separate sets of samples were used for the microbiological analyses on each sampling day. Samples were aseptically collected by excising 25g with a sterilized scalpel, of the top 0.5 cm of surface tissue. Samples were homogenized for 2 min in a sterile bag containing 225 mL of sterile 0.1% peptone water in a Stomacher lab blender (Model 400; Tekmer™ Co., Cincinnati, OH).

To determine the presence of each of the three pathogens, direct plating onto selective media was done, as well as following a Most Probable Number (MPN) method to enumerate each pathogen in samples where the number of bacteria were too few to detect by direct plating. *Listeria monocytogenes* cells were enumerated by serially diluting samples in 0.1% peptone buffer, plating onto Modified Oxford Agar plates and incubating them for 48 hr at 35°C. Typical colonies were enumerated, identified by Gram stain, catalase, oxidase, tumbling motility at 25°C, and their identity confirmed by Micro-ID Listeria (Organon Teknika, Durham, SC). For samples where no listeriae were detected by direct plating, a three-tube MPN procedure with University of Vermont Modified broth (UVM, Difco) was followed (McClain and Lee, 1989). Growth from positive tubes was inoculated onto Modified Oxford Agar plates, incubated for 48 hr at 35°C, and typical colonies were identified.

Yersina enterocolitica cells were enumerated by serially diluting samples as described, plating onto Cefsulodin-Irgasan-Novobiocin (CIN, Difco) agar plates and incubating these for 48 hr at 35°C. Typical red "bullseye" colonies with transparent borders were enumerated, identified by Gram stain, LAIA (LIA + arginine) slants and their identity confirmed by Enterotube II (Roche Diagnostic Systems, Montclair, NJ). For samples where no yersiniae were detected by direct plating, a three-tube MPN procedure with peptone sorbitol bile broth (PSBB, Difco) was followed (Wauters et al., 1988). Growth from positive tubes was inoculated onto CIN plates, incubated for 48 hr at 35°C, and typical colonies were identified.

Escherichia coli serotype O157:H7 cells were enumerated by serially diluting the samples, plating onto Sorbitol MacConkey agar (SMA, Difco) plates and incubating for 48 hr at 35°C. Typical colonies (red or colorless) were counted and their identity confirmed by a commercial agglutination test using O157 antiserum (Oxoid, Unipath Co., Columbia, MD). For samples where no *E. coli* O157:H7 were detected by direct plating, a 3-tube MPN procedure with EC broth (Difco) was followed (Doyle and Shoeni, 1987). Growth from positive tubes was inoculated onto SMA plates, incubated for 48 hr at 35°C, and typical colonies were identified as above

In most inoculated samples, cell counts were high (as expected) and could be enumerated by direct plating. Serial dilutions were prepared according to recommended microbiological procedures (Vanderzant and Splittstoesser, 1992), with 0.1 mL surface plated onto Plate Count Agar (PCA, DIFCO), and incubated aerobically at 25°C for 48 hr to enumerate total plate counts.

Sensory color and odor evaluation

Only uninoculated samples were used for color and odor evaluations that were conducted at day 0. A ten-member sensory panel evaluated samples by using a 5-point descriptive scale for surface color (1 = dark brown; 5 = dark red) and odor (1 = pleasant, no off-odor; 5 = extreme off-odor). Panelists were selected from faculty and graduate students in the Animal Science Department who were experienced with sensory panels. No training of panelists was included. Samples were tempered at room temperature (≈ 23°C) for 2 hr, unwrapped, and placed in Ziploc® (DowBrands L.P., Indianapolis, IN) pouches for evaluation. Ten steaks or five ground beef samples were evaluated by each panelist at each session. Steaks and ground beef were evaluated in separate sessions.

Statistical analyses

The experiment with steaks included 40 treatment combinations: two packaging treatments, four inoculation treatments, and five irradiation treatments (2 × 4 × 5 = 40). Measurements of pH, TBA, Hunter color L, a, b, and sensory evaluation were conducted only on uninoculated samples thus including 10 (2 × 1 × 5) treatment combinations. Microbiological, physical and chemical analyses were conducted at day 0, 7,

and 9; and sensory evaluation was conducted only at day 0. Three replications were conducted. The total number of steaks used was 480 [(40 × 3 + 10 × 3 + 10 × 1) × 3]. The experiment with ground beef included 15 treatment combinations: four inoculation treatments, and five irradiation treatments (4 × 5 = 20). The same sampling design was used as for steaks; therefore, the total number of ground beef samples was 240.

Microbiological data were transformed into logarithms of the number of colony-forming units/g ($\log_{10}$ CFU/g). Average data and standard errors were calculated from three replications. Each sampling time (day 0, 7, and 9) was analyzed separately because of the different temperatures used for storage. Physical, chemical, microbiological, and color/odor data were analyzed by using the Statistical Analysis System (SAS Institute Inc., 1986). The analysis of variance (ANOVA) procedure was used to detect the significances of replications, packaging (loins and steaks only), dose, and packaging by dose (loins and steaks only). Dose rate and other combination effects were also evaluated. If no significant difference existed between low and high dose rates, data were averaged for analyses of irradiation effects. Comparisons of means was based on Duncan s multiple range test.

RESULTS & DISCUSSION

Dose rate effects on microorganisms and quality attributes

Some dose-rate effects on microorganisms were observed (data not shown). Differences, however, were all < 2 log and were not consistent, with both high and low dose rates resulting in lower cell counts. For other attributes, no dose rate effect (P > 0.05) was found among samples (data not shown). Using a single radiation source means that dose-rate differences which can be obtained are relatively limited, although there was more than a twofold difference between low and high dose rates in this study. Further studies using a wider range of dose rates are needed to fully evaluate this.

Effects of irradiation on microorganisms

Listeria monocytogenes. Irradiation reduced the number of *L. monocytogenes* in both beef steaks and ground beef. In steaks, there was 1 log cell reduction of *L. monocytogenes* after low-dose (0.6 kGy) irradiation, compared with 3 log cell reduction achieved by medium dose (1.5 kGy) (Fig. 1-A, B). Storage at 7°C was effective in suppressing the growth of this organism in all samples, with no increasing counts observed during the storage period. Also, cell counts of irradiated steaks were lower than those of controls throughout storage, especially for medium-dose-treated samples. However, after temperature abuse, growth rates (Fig. 1-A, B) of cells exposed to medium dose were somewhat faster than the low dose treatments. Patterson et al. (1993) reported that irradiated cells showed an extended lag phase before growth which was directly related to irradiation dose. In our study, the "abused" samples were held at 25°C whereas Patterson et al. (1993) studied temperatures up to 15°C. Possibly the higher temperature and a 48 hr interval masked any lag phase difference that existed.

Varabioff et al. (1992) showed that *L. monocytogenes* survived well in chicken stored under vacuum or modified atmosphere (increased CO_2) compared with samples packaged in air after both were irradiated at 2.5 kGy. Grant and Patterson (1991) found that modified atmosphere (25% CO_2, 75% N_2) increased D_{10} values for *L. monocytogenes* and *Y. enterocolitica.* In our study, no significant difference was observed in number of survivors of *L. monocytogenes* regardless of storage atmosphere. This may be related to lower (0.60 or 1.5 kGy) doses, which allowed more total survivors.

There was a significant difference in number of survivors in ground beef as result of the dose level (Fig. 1 C). Low-dose (0.80 kGy) and medium-dose (2.0 kGy) irradiation reduced the number of *L. monocytogenes* by 1.5 log and 5 log, respectively. No obvious growth was observed during 7°C storage for any samples. Medium-dose irradiation decreased the number of *L. monocytogenes* cells to < 1 log CFU/g, and no survivors were detected after the temperature was elevated to 25°C. Growth of

this organism was totally masked by the large number (7.0 log CFU/g) of mesophilic organisms that grew in the ground beef at 25°C. Comparing the effect of medium-dose irradiation on survival of *L. monocytogenes* in steaks (1.5 kGy) vs ground beef (2.0 kGy), we found that irradiation at 2.0 kGy was effective for reducing this pathogen in both products, but surviving cells could proliferate during temperature abuse. Knabel et al. (1990) showed that storage under anaerobic conditions resulted in an increase in Listeria spp. survivors after a heat treatment compared with storage in air. In our study, MOX plates were incubated aerobically; thus, cell counts of this organism may have been less than if the plates had been incubated anaerobically. However, there was no significant change in cell counts during 7 days storage in vacuum or air atmospheres. This may be explained by the relatively short storage period; thus, no recovery effect due to atmospheric change was observed.

Yersinia enterocolitica. There was no significant difference in growth of *Y. enterocolitica* on steaks, regardless of packaging atmosphere (Fig. 2-A, B). This organism was reduced to undetectable levels by 1.5 kGy irradiation, and no further growth was observed during storage. Irradiation at 0.60 kGy suppressed cell growth only at the beginning of storage, with growth observed during extended storage. Hanna et al. (1976) reported that no *Y. enterocolitica*-like organisms were isolated until 21 days storage at 1–3°C and that isolation of these organisms was more frequent after 28 days storage under vacuum conditions than under nonvacuum. We found no difference for cell recovery between samples packaged under vacuum vs. air, again probably because of the relatively short storage.

In ground beef, almost all *Y. enterocolitica* cells were eliminated by irradiation, regardless of dose (Fig. 2-C). Low survival probably was due to that organism's poor ability to compete with the proliferating mesophiles, as well as its sensitivity to irradiation.

Although swine are considered a major natural reservoir for pathogenic *Y. enterocolitica*, contamination of beef with the organism is also possible (Ibrahim and Mac Rae, 1991; Andersen et al., 1991). However, the occurrence of *Y. enterocolitica* is usually low because this organism is less competitive than others found in animals. In this study, only two nonirradiated steaks were positive for *Y. enterocolitica* after 7 days storage. No irradiated steak was positive for *Y. enterocolitica* at day 0, even when doses as low as 0.6 kGy were used. Results confirmed those of El-Zawahry and Rowley (1979), who reported that a dose of 1 kGy was adequate to eliminate *Y. entercolitica* in raw meat.

Escherichia coli O157:H7. *E. coli* O157:H7 was completely eliminated by irradiation at 1.5 kGy, with no survivors detected on steaks during storage (Fig. 3-A, B). There was at least 1 log reduction by 0.6 kGy irradiation, but the cells continued to grow during storage at 7°C and 25°C. Thus, low-dose irradiation could provide increased safety for products but only if they were not heavily contaminated. This organism is usually present in meat in low numbers, with < 15 organisms/g (USDA, 1993) reported. After temperature abuse, growth of cells in the control samples was suppressed to undetectable levels, regardless of storage atmosphere. This may have resulted from accumulated CO_2 in packages, which was toxic to this organism. Spoilage occurred after 2 days at the abuse temperature, regardless of packaging method, as evidenced by total plate count of 10^7 cells/g (data not shown). No effect of air vs. vacuum packaging was found in this study which is consistent with a study by Thayer and Boyd (1993). They also reported that growth of *Salmonella* spp. (Thayer and Boyd, 1991) was not affected by air in the storage environment when irradiated in mechanically deboned chicken meat. They suggested that the mass of the meat probably masked any possible oxygen effect on the cells.

In ground beef, *E. coli* O157:H7 was also eliminated by medium-dose (2.0 kGy) irradiation, and not many cells were recovered during storage at 7° or 25°C (Fig. 3-C). There was only a 2-log cell reduction after irradiation at 0.8 kGy. Cell number

Fig. 1—Growth of *L. monocytogenes* in air (A) or vacuum (B) packaged steaks and ground beef (C) treated with irradiation and stored at 7°C (day 0–7) followed by 25°C (day 7–9). Bars represent standard deviation of triplicates.
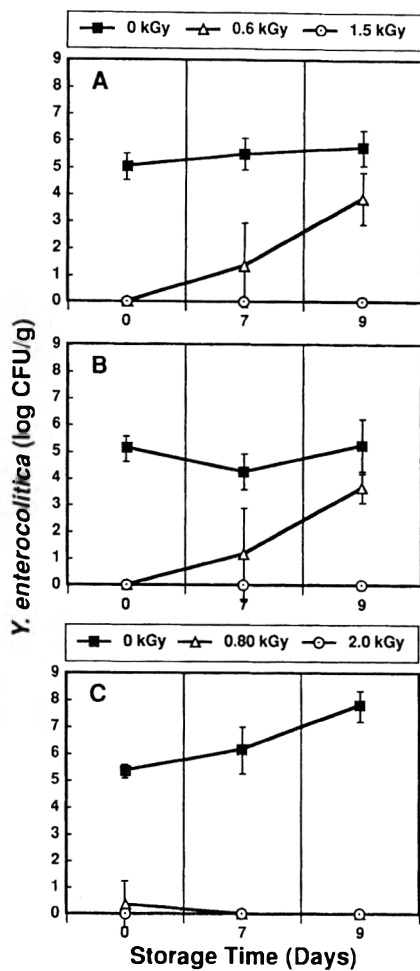
Fig. 2—Growth of *Y. enterocolitica* in air (A) or vacuum (B) packaged steaks and ground beef (C) treated with irradiation and stored at 7°C (day 0–7) followed by 25°C (day 7–9). Bars represent standard deviation of triplicates.
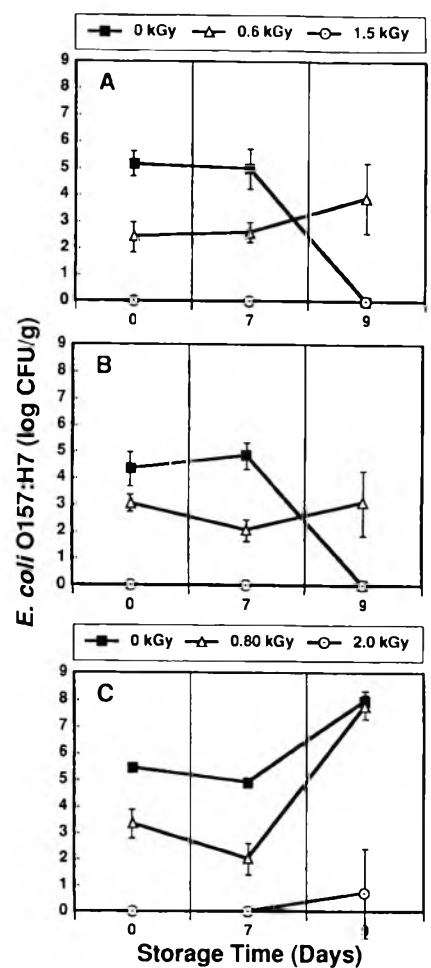
Fig. 3—Growth of *E. coli* O157:H7 in air (A) or vacuum (B) packaged steaks and ground beef (C) treated with irradiation and stored at 7°C (day 0–7) followed by 25°C (day 7–9). Bars represent standard deviation of triplicates.

was decreased during storage at 7°C, but this organism proliferated rapidly after temperature abuse and reached the same number of survivors as for controls. More than half the outbreaks of *E. coli* O157:H7 infections have been attributed to ground beef (Doyle, 1991; Mermelstein, 1993); irradiation at 2.0 kGy can significantly decrease the risk of *E. coli* O157:H7.

When medium doses were applied, *Y. enterocolitica* and *E. coli* O157:H7 were both reduced to undetectable levels, whereas some *L. monocytogenes* cells still survived (Fig. 1-3). These results confirmed that *L. monocytogenes* were more resistant to irradiation than were the other two pathogens (Thayer, 1993).

Uninoculated samples. The effect of irradiation on uninoculated samples was also monitored. A small number of steaks were found to contain *Y. enterocolitica*, but no *L. monocytogenes* or *E. coli* O157:H7 were found in any uninoculated samples. Enumeration of TPC as an index of total microbial load in meat is often used as a routine check for quality-control. From our results, TPC were reduced by 2-3 log immediately after irradiation at 1.5 kGy in steaks packaged under vacuum or in air (Fig. 4–A, B). Samples irradiated at 0.60 kGy resulted in a reduction in TPC of 1 log in vacuum-packaged steaks but in 2 log when packaged in air. This suggests that irradiation may be more effective for reducing TPC when oxygen is present, possibly due to an increase in formation of oxygen-related free radicals, which can affect microorganisms. Dose effect during 7°C storage was significant (P < 0.05), and the medium dose was more effective. However, after temperature abuse there was

no difference (P > 0.05) between any of the samples, regardless of dose. It is possible that recovery occurred more quickly in low-dose abused samples but after 2 days abuse no difference was observed. Thus, fresh beef irradiated at doses up to 1.5 kGy still required adequate refrigeration to ensure safety and long shelf life.

The initial TPC of ground beef was 7.0 log CFU/g (Fig. 4–C), which was higher than for beef steaks (3.5 log CFU/g). Low (0.80 kGy) and medium (2.0 kGy) doses of irradiation caused 1.5- and 3.0-log reduction of TPC, respectively. When stored at 7°C, TPC of irradiated samples increased but remained < 7.0 log CFU/g. Nonirradiated samples on the other hand, had already spoiled (reached 8.5 log CFU/g) after 7 days storage at the same temperature. After 2 days storage at 25°C, samples irradiated at 0.80 kGy also reached spoilage levels, while samples irradiated at 2.0 kGy were at the threshold (7.0 log CFU/g). This indicated that meat samples with higher microbial loads such as ground meat, required a higher dose of irradiation to effectively reduce contaminants and extend shelf life.

Low temperature and vacuum packaging are very important to maintaining high microbiological quality of meat. Longer shelf life can be obtained when beef steaks are stored at lower temperature (4°C or lower) than 7°C. Lebepe et al. (1990) reported that 3.0-kGy radiation extended the microbiological shelf life of vacuum-packaged pork loins stored at 2-4°C to > 90 days. In our study, no significant difference (P > 0.05) in TPC was observed between packaging environments when stored at 7°C, but storage time was relatively short (7 days).
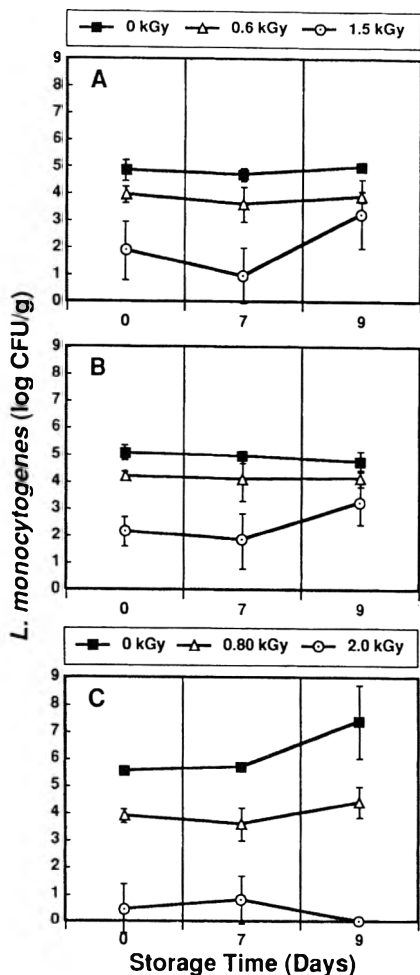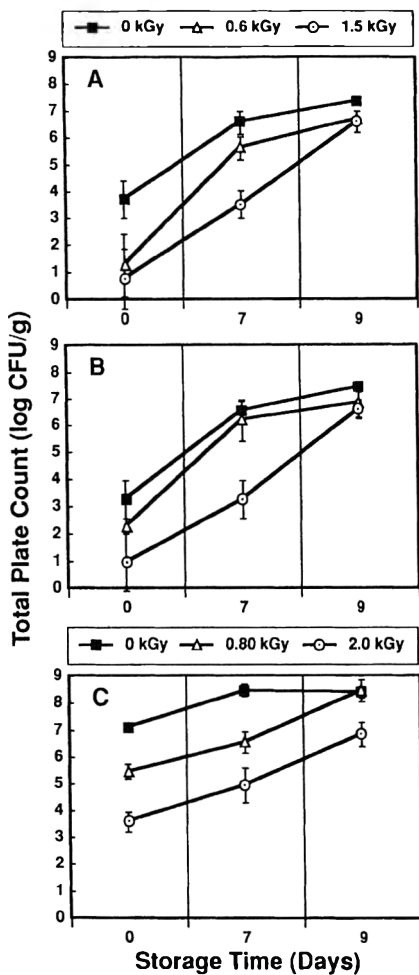
Fig. 4—Total plate counts in air (A) or vacuum (B) packaged steaks and ground beef (C) treated with irradiation and stored at 7°C (day 0–7) followed by 25°C (day 7–9). Bars represent standard deviation of triplicates.

Irradiation effects on quality attributes

No pH differences were observed in steaks (Table 1) or ground beef (Table 2) until after 9 days storage and then probably resulted from microbial proliferation.

TBA values for steaks increased after 7 days storage for all samples, with in-air packaging resulting in more lipid oxidation than vacuum packaging, as we expected. TBA values were not positively correlated with microbial load. Microbial degradation of malonaldehyde and other thiobarbituric acid reactive substances (TBARS) (Moerck and Ball, 1974) may have occurred. TBA values for ground beef were > 2.0 at day 0, and there was no significant difference (P > 0.05) in values between treatments (Table 2). After 7 or 9 days storage, irradiated samples showed higher TBA values than controls. Irradiation may have delayed microbial degradation of malonaldehyde and other TBA reactive substance, resulting in higher TBA values than those of the control. However, the validity of using TBA as an index for lipid oxidation of meat products after irradiation and during storage needs to be further investigated.

No significant (P > 0.05) color difference for steaks was observed by the Hunter method (Table 1). Also, sensory evaluation showed no color difference (P > 0.05) between control and irradiated samples (Table 3). Regardless of packaging methods irradiated samples were not different than controls for off-odor scores. However, some panelists noted off-odors in irradiated samples. Because samples were evaluated 2 hr after packages were opened, dissipation of odor may have occurred. Vacuum-packaged beef often develops unique odors even in the absence of a high level of contaminating microorganisms (Egan and

Table 1—Selected characteristics of irradiated steaks packaged in air (A) and in vacuum (V) during storage at 7°C (day 0–7) and 25°C (day 7–9)

| | Days of storage | Irradiation dose (kGy) | | | | | |
| | | 0.0 | | 0.60 | | 1.5 | |
		A	V	A	V	A	V
pH	0	5.51[a]	5.58[a]	5.51[a]	5.55[a]	5.53[a]	5.62[a]
	7	5.51[a]	5.48[a]	5.57[a]	5.45[a]	5.53[a]	5.51[a]
	9	5.23[c]	5.31[bc]	5.35[ab]	5.30[bc]	5.43[a]	5.42[a]
TBA	0	0.65[a]	0.27[ab]	0.42[ab]	0.14[b]	0.58[ab]	0.15[b]
	7	2.33[a]	0.76[b]	2.43[a]	1.23[b]	2.58[a]	1.25[b]
	9	1.84[a]	0.71[b]	2.16[a]	1.02[ab]	2.07[a]	1.03[ab]
L value	0	30.0[a]	28.8[a]	29.3[a]	23.3[a]	29.6[a]	27.2[a]
	7	28.3[a]	28.5[a]	29.6[a]	23.6[a]	29.1[a]	29.1[a]
	9	30.8[a]	31.3[a]	29.7[a]	29.9[a]	29.5[a]	29.0[a]
a value	0	9.4[a]	6.2[c]	7.5[b]	6.5[c]	6.4[c]	5.9[c]
	7	7.3[a]	7.1[a]	7.1[a]	7.0[a]	6.7[a]	6.6[a]
	9	6.9[a]	7.0[a]	6.4[ab]	6.8[a]	6.0[b]	6.5[ab]
b value	0	5.8[a]	4.6[b]	4.8[b]	4.0[b]	4.8[b]	4.1[b]
	7	5.4[ab]	4.4[c]	4.8[abc]	4.6[bc]	5.5[a]	4.8[abc]
	9	4.6[a]	4.7[a]	5.1[a]	4.7[a]	4.7[a]	4.5[a]

[a-c] Values within each row with the same superscripts are not significantly different (p > 0.05).

Table 2—Selected characteristics of irradiated ground beef during storage at 7°C (day 0–7) and 25°C (day 7–9)

| | Days of storage | Irradiation dose (kGy) | | |
		0.0	0.80	2.0
pH	0	5.53[a]	5.48[a]	5.52[a]
	7	5.46[a]	5.25[a]	5.29[a]
	9	6.51[a]	5.63[b]	5.52[d]
TBA	0	2.23[a]	2.08[a]	2.08[a]
	7	2.27[b]	4.01[a]	4.30[a]
	9	2.22[b]	5.28[a]	6.19[a]
L value	0	43.8[a]	42.4[b]	42.4[b]
	7	44.3[a]	45.4[a]	44.3[b]
	9	43.7[a]	45.6[a]	41.9[a]
a value	0	8.1[a]	7.7[ab]	7.0[b]
	7	6.9[a]	6.5[a]	6.4[a]
	9	6.0[b]	7.1[a]	6.2[ab]
b value	0	7.6[a]	6.8[b]	6.5[b]
	7	7.1[a]	7.4[a]	6.8[a]
	9	6.5[b]	7.6[a]	7.9[a]

[a,b] Values within each row with the same superscripts are not significantly different (p > 0.05).

Table 3—Sensory color and odor characteristics of irradiated steaks packaged in air (A) and in vacuum (V)

| | Irradiation dose (kGy) | | | | | |
| | 0.0 | | 0.60 | | 1.5 | |
	A	V	A	V	A	V
Color	3.9[a]	4.1[a]	3.4[t]	3.9[a]	2.4[c]	3.6[ab]
Odor	1.2[b]	1.5[b]	2.0[a]	2.2[a]	2.4[a]	2.2[a]

[a-c] Values within each row with the same superscripts are not significantly different (p > 0.05).

Shay, 1982) due to predominantly lactic acid bacteria. After irradiation, lactic acid bacteria were predominant and may have contributed some odor development. For ground beef, although some values for L, a ,b color measurements were different (P < 0.05) (Table 2), no differences (P > 0.05) in sensory color or odor were detected by panelists (Table 4).

CONCLUSIONS

FRESH BEEF, especially ground beef, is easily contaminated during processing. Irradiation, followed by refrigeration was found to be a very effective way to reduce initial microbial loads, improve safety and extend shelf life of meat without affecting sensory quality. Elimination of pathogens was directly related

Table 4—Sensory color and odor scores of irradiated ground beef

	Irradiation dose (kGy)		
	0.0	0.80	2.0
Color	3.2[a]	3.0[a]	2.8[a]
Odor	3.1[a]	2.8[a]	3.0[a]

[a] Values within each row with the same superscripts are not significantly different (p > 0.05).

to total irradiation dose, but dose rate was not a factor affecting survival of microorganisms.

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Ms received 11/3/94; revised 5/15/95; accepted 5/21/95.

Ground Beef Characteristics after Browning or Oil Extraction Preparation

K. S. RHEE and M. L. BRAVO-GUTIERREZ

──────────── ABSTRACT ────────────

Ground beef with 26.3% fat was cooked by browning/draining (BD), browning/rinsing (BR, and an oil-extraction method with (OA) or without (ON) meat broth added back. BR caused the most reduction of total fat and cholesterol, whereas ON and OA yielded less cooked meat than BD and BR. ON and OA cooked samples, compared to BD and BR samples, contained more polyunsaturated fatty acids and nonheme iron and had higher TBA values upon extended storage at 4°C. Trained sensory panelists rated BD or BR cooked ground beef more intense in positive flavor note ("cooked beef/brothy") than ON or OA meat. Likewise, consumers tended to prefer meatloaf, spaghetti sauce, and chili prepared with BD or BR cooked ground beef to those prepared with ON or OA cooked meat.

Key Words: ground beef, stir frying, nutrients, sensory, lipid oxidation

INTRODUCTION

GROUND BEEF has been a favorite food among many consumers. Ground beef and its products accounted for 42% of total beef consumption in 1990 (AMI, 1991). In the home, browning— cooking crumbled meat to brown the product (and render out the fat from high-fat meat)—is the most common ground beef preparation method, other than cooking beef patties. Mixed recipes using ground meat as a major ingredient often involve browning the meat and draining the cooked-out liquid before adding other ingredients. An additional step, such as rinsing the browned-drained meat with warm or hot water, can be taken to remove residual fat on the surface of cooked meat particles (Love and Prusa, 1992; Snyder et al., 1992).

Small et al. (1991) proposed an unconventional ground meat cooking method to make the meat more healthful; a patent (U.S. 4,980,185) was granted for the method. Ground meat was heated in unsaturated (vegetable) oil to 100°C or higher, strained, and finally rinsed with boiling water. Their results showed that total fat, saturated fatty acids, and cholesterol in ground beef ($\approx$21% fat) were reduced more by oil extraction/rinsing than by browning/rinsing or cooking as patties. However, the researchers did not document sensory properties of the resultant meat or food products prepared with such meat. Additionally, storage stability of the oil extracted-rinsed meat was not determined. Our objective was to compare the oil extraction method with other cooking methods for loose or crumbled (not formed into patties) ground beef, with regard to nutrient retention in cooked meat, sensory properties of cooked meat (with no other ingredients) as well as mixed food products/dishes prepared with such precooked meat, and lipid oxidation in the precooked meat during storage.

MATERIALS & METHODS

Meat materials

Fresh beef chucks were obtained from the Texas A&M University Rosenthal Meat Science and Technology Center. Manually separated fat

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and lean fractions were ground separately through a grinder plate with 1.27 cm-diameter holes and subsampled for fat analysis using a CEM Automatic Extraction System with Automatic Volatility Computer (CEM Corp., Indian Trail, NC). The ground lean and fat fractions were mixed in appropriate proportions to produce ground beef batches with target fat levels of 8% and 25%, and reground through a grinder plate with 0.32 cm-diameter holes. Fat levels of the two batches were 9.0% and 26.3%, respectively (Table 1). Each batch was divided into 1,500-g portions, vacuum-packaged, and frozen (<1 week) at −20°C until used for cooking or raw meat analyses.

Ground beef cooking treatments

Of the following five treatments, only one (B) utilized the low-fat (9.0%) ground beef, with the other four using the high-fat (26.3%) meat. Before cooking, vacuum-packaged frozen ground beef was thawed, without breaking the vacuum seal, at 4°C for 24 hr. Four cooking replications (1,500g meat/replication) per treatment were conducted for cooking yield determination, compositional analyses, and storage experiments. Sensory evaluation samples were cooked separately.

Browned (B). Ground beef at 4°C was manually crumbled and browned (without added oil) for 16 min, with constant stirring, in a Teflon-coated electric skillet (Model 72010, West Bend Co., Manitowoc, WI) set at 150°C with no preheating. At the completion of browning, temperature of the center of the meat mass (piled-up cooked meat) reached $\approx$79°C as measured by a thermocouple. It was easier to brown the meat uniformly at 150°C than at a higher skillet temperature (Rhee et al., 1993).

Browned and drained (BD). Ground beef at 4°C was browned for 16 min as described. The meat was divided into two equal portions and each was drained 5 min through a funnel (17-cm diameter at the top) lined with one layer of cheesecloth. The drip was discarded.

Browned and rinsed (BR). Ground beef at 4°C was browned and drained as described for BD, and then the cooked meat (equivalent to 750g raw meat) in each funnel was rinsed with 187.5 mL boiling water.

Oil-extracted/no broth added (ON). This procedure and the subsequent one (OA) were adapted from those of Small et al. (1991). Crumbled ground beef (1,500g) was heated with 750 mL soybean oil (containing no additives), with constant stirring, until the meat-oil mixture reached $\approx$90°C. [The cold (4°C) meat and oil took $\approx$12 min to reach $\approx$90°C in a skillet set at 150°C with no preheating]. The mixture was stirred for 5 min with the heat turned off; temperature of the mixture remained at about 90°C during this time. The heat was turned back on and the mixture was stirred another 15 min, until its temperature reached $\approx$105°C. The heat was turned off and the mixture was allowed to stand for 3 min. The meat-oil mixture was divided into two equal portions, drained, and rinsed with boiling water, as described for BR. The rinsed meat was placed in a plastic bowl with the cover on and held at 4°C for 80 min. The holding was done to more accurately compare this version (ON) of the oil-extraction method with the next, broth added version (OA).

Oil-extracted/broth added (OA). Ground beef was cooked/extracted with soybean oil, drained, and rinsed with boiling water, as described for ON. However, the meat cook-out and oil from the oil-extraction step and liquid from water rinsing were collected in a Pyrex baking dish (28 × 18 × 4 cm) placed on ice and held in a −20°C freezer for 60 min. The solidified fat was lifted off as a cake and the aqueous (broth) layer was boiled in a Pyrex beaker for $\approx$20 min, to $\approx$9 mL. The concentrated broth was added back to the cooked meat (from 1500g raw meat) that had been held at 4°C during broth separation and concentration.

Use of cooked meat in mixed recipes

Three products varying in spice intensity and flavor—meatloaf, spaghetti sauce, and chili—were prepared using the ground beef from BD,

Table 1—Cooking yield, proximate composition, and cholesterol values for ground beef prepared by different methods

Cooking[e] method	Cooking yield (%)	Moisture (g/100g)		Protein (g/100g)		Total fat (g/100g)		Cholesterol (mg/100g)		Ash (g/100g)	
		Raw	Cooked	Raw	Cooked	Raw	Cooked	Raw	Cooked	Raw	Cooked
B	85.3[a]	71.0[a]	67.9[a]	20.1[a]	23.8[c]	9.0[b]	9.9[c]	55.2[b]	77.0[b]	1.0[a]	1.1[a]
BD	60.6[b]	56.3[b]	59.5[b]	15.8[b]	27.3[ab]	26.3[a]	14.1[a]	68.1[a]	90.3[a]	0.7[b]	1.1[a]
BR	58.0[c]	56.3[b]	60.4[b]	15.8[b]	26.0[b]	26.3[a]	12.2[b]	68.1[a]	79.6[b]	0.7[b]	0.9[b]
ON	56.6[d]	56.3[b]	57.8[c]	15.8[b]	28.1[a]	26.3[a]	14.0[a]	68.1[a]	95.4[a]	0.7[b]	1.0[a]
OA	57.5[d]	56.3[b]	57.8[c]	15.8[b]	26.7[ab]	26.3[a]	14.0[a]	68.1[a]	94.6[a]	0.7[b]	1.1[a]

[a-d] Means within the same column with the same superscript are not significantly different (p>0.05).

[e] B used low-fat (9.0%) raw ground beef; BD, BR, ON and OA used high-fat (26.3%) ground beef.

BR, ON, or OA treatment. Small et al. (1991) stated that they used their oil-extracted ground meat in various recipes (including meatloaf, spaghetti sauce, and chili con carne) that called for ground or finely chopped meat, but presented no sensory data. Recipes from a ground beef cookbook (Southern Living, 1972) were modified through preliminary experiments. Nonmeat ingredients used for each product and their quantities (based on precooked meat from 1500g raw meat) are specified.

Meatloaf. Two eggs ("large" size), 10g salt, 120g plain bread crumbs, 153g 2%-fat fluid milk, 60g finely chopped fresh celery, and 60g finely chopped fresh onion were used. The meatloaf mixture was divided into two equal portions (for 2 loaves), placed in aluminum pans (20.3 × 9.8 × 6.0 cm) with the top leveled, baked in a 204°C preheated oven for 20 min, and cooled 15 min at room temperature and another 15 min at 4°C. Loaves were vertically sliced into individual serving sections, each weighing ≈20g; the browned surface was removed from each loaf for uniformity of sliced sections. The meatloaf slices were placed in either tops or bottoms of Pyrex petri dishes (5-cm diameter). The dishes were then placed on styrofoam trays (6/tray), vacuum-packaged, and held at −20°C until the next day for sensory evaluation.

Spaghetti sauce. Canned tomato sauce (800g), 173g chopped fresh onion, 5g crushed fresh garlic, 30g Worcestershire sauce, 15g salt, 3.5g oregano (dried), 5g black pepper, and 10g sugar were added to precooked ground beef. The sauce mixture was simmered for 20 min, divided into 20g portions, and handled in the same manner as meatloaf samples.

Chili. Nonmeat ingredients—185g chopped fresh onion, 10g crushed fresh garlic, 80g fresh green bell pepper (blanched and chopped), 467g canned chili beans, 750g canned tomato sauce, 500g tomato catsup, 4g white vinegar, 20g prepared mustard, 8g chili powder, 15g hot sauce, 60g flour, and 15g salt—were combined with precooked meat, simmered for 20 min, and handled in the same manner as meatloaf samples.

Determination of cooking yield and nutrient retention

Cooking yield (%) was determined by meat weight difference before and after B, BD, BR, ON, or OA treatment. Retention of a meat component/nutrient, as a percentage of the amount in raw meat, was calculated according to the procedure outlined by Murphy et al. (1975). Total food energy (kcal) for cooked ground beef was calculated from fat and protein contents (9 kcal/g fat; 4 kcal/g protein) since the amount of carbohydrate in beef is negligible (USDA, 1990).

Chemical analyses

Moisture (by oven drying), ash, and crude protein were determined in triplicate by AOAC (1990) procedures. Total lipid was extracted according to the procedure of Folch et al. (1957) and fat content was gravimetrically determined in triplicate on aliquots of extract after solvent removal. Cholesterol content was determined as described by Bohac et al. (1988).

Total iron was determined on wet-ashed (Parkinson and Allen, 1975) samples by flame atomic absorption spectrometry. The modified Schricker nonheme iron method (Rhee and Ziprin, 1987) was used for nonheme iron analysis. Heme iron content was estimated by subtracting nonheme iron values from total iron values.

An aliquot of the total lipid extract was transmethylated by the procedure of Metcalfe and Wang (1981) and fatty acid methyl esters were analyzed by gas chromatography as described by Rhee et al. (1988). Weights (g) of individual fatty acids/100g sample were estimated from fatty acid weight percentage data used in conjunction with total fat content (Rhee, 1994).

Lipid oxidation was determined using a distillation TBA procedure with an antioxidant solution (propyl gallate-EDTA) added at the blending step (Rhee, 1978). From each cooking replication of BR, BD, ON, or OA, 390g cooked meat was divided into 65g portions in 100-mL

beakers, over-wrapped with Saran® Wrap (Dow Consumer Products, Indianapolis, IN), and stored at 4°C for 0, 1, 2, 4, 6, or 8 days. Each stored sample (60g) was blended with 30 mL antioxidant solution and 90 mL distilled-deionized water, and two 30g portions of the slurry were distilled.

Sensory analyses

Cooked ground beef samples (with no added ingredients) of BD, BR, ON, and OA treatments were evaluated by a trained sensory panel, whereas samples of meatloaf, spaghetti sauce, and chili made with ground beef precooked by BD, BR, ON, or OA were evaluated by consumer panels. Panelists were seated in individual sensory booths and received samples through hatches of a bread-box type.

Evaluation of cooked ground beef samples. Five highly trained and experienced panelists were used. They were professional panelists at the Texas A&M University Sensory Testing Facility who had been originally trained according to the Spectrum™ procedures of Meilgaard et al. (1991) and had evaluated various meat products over many years with continuous retraining. Prior to test sessions on the four cooking treatments, a product orientation/retraining session lasting ≈2 hr was held with reference products for Spectrum™ intensity scales and some cooked beef samples, such as browned ground beef and pan-broiled beef steaks (the lean and fat). The following flavor attributes (aromatics) were evaluated on a 0–15 intensity scale (0 = absent; 15 = extremely intense): cooked beef/brothy, cooked beef fat, serum/bloody, grainy/cowy, cardboard, painty, fishy, liver, soured/grainy, and browned.

The meat samples were cooked in triplicate for each cooking treatment (1500g raw meat/cooking replication/treatment) a day before evaluation, portioned into individual servings, vacuum-packaged, and held overnight at −20°C, as described earlier. On the following morning (sensory evaluation day), frozen samples (still vacuum-packaged) were tempered at room temperature for 1 hr before reheating. Five servings (in petri dishes) at a time were reheated in a microwave oven (700 watts) at "high" power for 1.5 min. The bottom of each dish was checked with the hand for uneven heating, and any inadequately heated dish was microwaved for an additional 5–10 sec (5 sec at a time) until warm.

Two mid-morning test sessions were held on the same day; six samples were served in each session with a 15-min break between sessions. Two samples (two cooking replications) of BD, two BR, one ON, and one OA were evaluated in the first session and two ON, two OA, one BD, and one BR in the second session. Sample serving order in each session was random. Unsalted crackers and water were provided for cleansing/rinsing between samples. Samples were evaluated under red light to avoid bias due to color.

Consumer evaluation of mixed recipe products. Untrained panelists (at least 50 for each product)—students, staff, and faculty at Texas A&M University—evaluated the meatloaf, spaghetti sauce, and chili samples, with each product evaluated on a separate day. Samples were evaluated for flavor, texture, and overall acceptability on an 8-point hedonic scale (from 1 = dislike extremely to 8 = like extremely, with all of the 8 points given descriptors). Vacuum-packaged individual servings held overnight at −20°C were tempered and reheated as described for cooked ground beef samples. Cooking of the ground beef (by BD, BR, ON, or OA) in preparation of each mixed recipe product was not replicated. Panelists were asked to proceed at their own pace and were provided with a cup of water to rinse between samples, which were evaluated under red light.

Statistical analysis

Data were evaluated by analysis of variance and mean separation by the Student-Newman-Keuls test. Correlation procedure was used to determine relationships between experimental variables. The SAS Institute,

Table 2—Retention of moisture, protein, fat, cholesterol, and ash in ground beef cooked by different methods

Cooking[e] method	Retention (%)				
	Moisture	Protein	Total fat	Cholesterol	Ash
B	82[a]	101[ab]	95[a]	119[a]	99[a]
BD	64[b]	105[a]	33[b]	80[b]	90[b]
BR	62[c]	96[b]	27[c]	68[c]	73[d]
ON	58[d]	101[ab]	30[b]	79[b]	81[c]
OA	59[d]	97[b]	31[b]	80[b]	82[c]

[a-d] Means within the same column with the same superscript are not significantly different (p>0.05).

[e] B used low-fat (9.0%) raw ground beef; BD, BR, ON and OA used high-fat (26.3%) ground beef.

Inc. (1990) program was used for data analysis. Significance was established at p ≤ 0.05 unless otherwise indicated.

The entire study consisted of four analytical/experimental components. One-factor analysis of variance was used for chemical composition data on raw samples at two fat levels. Cooked ground beef composition data and TBA values within each storage period were treated as one factor, randomized block experiments with five cooking treatments (B, BD, BR, ON, OA) blocked by cooking replications. A complete block design was used for trained sensory panel data on cooked ground beef samples with panelists serving as blocks. Consumer sensory data on meatloaf, spaghetti sauce, or chili samples were treated as three separate experiments of randomized design, each having four treatments (BD, BR, ON, OA).

RESULTS & DISCUSSION

Cooking yield

Among the five cooking treatments, B (browning of the low-fat meat) had the highest cooking yield because all the cook-out remained with the meat. Of the high-fat meat cooking methods, BD had the highest yield and ON and OA (the two variations of oil-extraction) the lowest (Table 1). The cooking yield of BD (≈61%) was similar to the ≈65% yield reported by Cannell et al. (1989) on ground beef with 25.42% fat and the ≈62% yield by Love and Prusa (1992) of ground beef with 27.3% fat.

ON and OA required the longest cooking times (>30 min, excluding the broth separation/concentration time for OA vs <17 min for BD or BR). The long preparation time is a major disadvantage for both versions of oil extraction since a meal taking 30 min or less is considered critical for consumers (NLSMB, 1992).

Proximate composition and cholesterol content

The low-fat ground beef used for B treatment, when compared to the high-fat ground beef used for BD, BR, ON, or OA treatment, was higher in moisture, protein, and ash and was lower in cholesterol (Table 1). When cooked, B samples had the highest moisture and contained the least fat/100g. Among the cooking techniques used for the high-fat meat, BR yielded cooked meat with the lowest fat content. Although BR, ON, and OA were subjected to draining and rinsing at some point during cooking/preparation process, ON and OA might have had some oil penetration into meat particles. The penetrated oil would not be removed by draining and rinsing. Protein values/100g cooked meat were lowest for B samples because the cook-out (consisting primarily of water) was allowed to remain with the meat for this treatment. ON and OA samples contained more cholesterol than BR samples. Total fat and cholesterol values correlated positively (r = 0.71; p < 0.001) when data on cooked samples of all five treatments were included. BR (219 kcal/100g) yielded cooked meat with lower total calories when compared to BD, ON, and OA (236, 238, and 232 kcal/100g, respectively). The low-fat B samples had the least caloric value (185 kcal/100g).

Retention as percentage of amount in raw meat

While cooked meat composition data (g/100g meat) are often used for dietary planning, percent retention data indicate true

effects of cooking on nutrients. B treatment on the low-fat meat resulted in higher moisture, fat, and ash retention than did those cooking treatments for high-fat meat (Table 2) because the cook-out was retained with the cooked meat for B, as mentioned. Among the high-fat meat cooking treatments, BD retained the most moisture and ON and OA the least. BR was most effective for lowering fat and cholesterol retention. In contrast, Small et al. (1991) reported that the oil extraction method was more effective than BR. In their study, oil extraction of ground beef containing 20.7% fat resulted in 32.3% fat retention (reported as 67.7% loss), similar to our ON and OA data (30% and 31% retention) on ground beef with 26.3% fat (Table 2); however, fat retention for BR was higher in their study (41% vs 27%). The large fat retention difference between our BR samples and their "stir-fried"-and-rinsed (corresponding to our BR) samples could be due largely to the low browning temperature they employed. The amount of boiling water used to rinse the cooked meat resulting from a given weight of raw meat was greater in their study (50 mL/100g vs 25 mL/100g). They browned ground beef at 100–110°C, a much lower temperature than browning temperatures used in other studies [i.e.. 150°C we used, 163°C by Cannell et al. (1989), and 177°C by Love and Prusa (1992)]; they did not specify browning time. A cookware temperature of 100–110°C (212–230°F) seems to be unusually low to brown ("stir-fry") ground meat. Browning ground meat (especially high-fat meat) is essentially a pan-frying process. The temperature of a cooking utensil, when controllable by a thermostat as for an electric skillet, is usually set at 149°C (300°F) or higher for pan-frying.

As for cholesterol, oil extraction of ground beef containing 20.7% fat resulted in 60.8% retention (reported as 39.2% loss) in the study by Small et al. (1991), which was lower than our cholesterol retention values for ON and OA (79% and 80%). Cholesterol retention for BR was higher in their study (81.7% vs 68%), as was fat retention.

Cooked meat compositional data reported by Love and Prusa (1992)—cooked meat nutrient data reported as amounts derived from 100g raw meat—were used to calculate retention percentages to compare with our data. Their BR samples from raw ground beef containing 27.3% fat (vs 26.3% fat in our study) retained 16% of the fat and 62% of the cholesterol present in raw samples, which were basically confirmed by our results. These researchers browned meat at 177°C (vs 150°C in our study), and the cooked meat from a 200g portion of raw meat (one-half of the cooked meat from 400g raw meat) was rinsed with 1000 mL water at 65–70°C. Although they used a larger volume of rinsing water per meat weight (raw basis), the temperature of their rinsing water was lower.

Moisture retention was lower for ON and OA than for BD or BR. This might be attributed to increased evaporation losses that could have occurred during oil extraction involving an extensive heat treatment and to the moisture replacing effect of cooking meat in oil. Protein retention was high (>95%) for all treatments because the protein in meat would have been coagulated by these cooking treatments and the coagulated protein would be unlikely to migrate into the drip or rinsing water.

BR resulted in the least ash retention among high-fat meat treatments and BD the most. Ash components, mostly minerals, are water-soluble. This water-solubility may explain the ash retention difference between BD and BR, i.e., the hot-water rinsing step in BR increased ash loss through a leaching effect. The oil used in the browning step of ON and OA might have had a protective coating effect and reduced losses of certain ash components in the rinsing step.

Nonheme iron (NHI) and heme iron

Cooked meat total iron content (3.1–3.4 mg/100g) or retention (80–88%) was not significantly different among high-fat meat treatments. All cooked samples, including those of B, had similar (p > 0.05) total iron values. ON and OA yielded cooked

Table 3—Nonheme iron and heme iron values and percent changes due to cooking for ground beef cooked by different methods

	Nonheme iron			Heme iron		
Cooking[d] method	Raw (μg/g)	Cooked (μg/g)	% Change[e] due to cooking	Raw (mg/100)	Cooked (mg/100g)	% Change[e] due to cooking
B	5.0[a]	10.8[c]	+ 84[b]	2.8[a]	2.1[a]	− 37
BD	3.8[b]	11.6[c]	+ 85[b]	1.9[b]	2.0[a]	− 34
BR	3.8[b]	12.2[b]	+ 85[b]	1.9[b]	1.9[a]	− 41
ON	3.8[b]	16.3[a]	+ 142[a]	1.9[b]	1.8[a]	− 45
OA	3.8[b]	15.8[a]	+ 138[a]	1.9[b]	1.8[a]	− 43

[a-c] Means within the same column with the same superscript are not significantly different (p>0.05).

[d] B used low-fat (9.0%) raw ground beef; BD, BR, ON and OA used high-fat (26.3%) ground beef.

[e] {[(cooked value/raw value) × (% cooking yield/100)] × 100} − 100.

Table 4—Total saturated (SFA), monounsaturated (MUFA), and polyunsaturated (PUFA) fatty acids for ground beef cooked by different methods

	% of total fatty acid			g/100g sample		
Meat	SFA	MUFA	PUFA	SFA	MUFA	PUFA
Raw						
Low-fat	45.7[a]	50.0[a]	4.4[b]	3.5[d]	3.8[b]	0.3[c]
High-fat	45.1[a]	52.0[a]	2.9[b]	10.4[a]	12.0[a]	0.7[b]
Cooked[e]						
B	43.2[a]	52.9[a]	3.9[b]	3.6[d]	4.4[b]	0.3[c]
BD	43.8[a]	46.0[a]	2.7[b]	5.3[b]	5.6[b]	0.3[c]
BR	43.1[a]	53.7[a]	3.2[b]	4.5[c]	5.6[b]	0.3[c]
ON	29.6[b]	43.4[a]	27.0[a]	3.6[d]	5.2[b]	3.3[a]
OA	31.6[b]	42.6[a]	25.8[a]	3.8[d]	5.1[b]	3.1[a]

[a-d] Means in the same column having the same superscript are not significantly different (p>0.05).

[e] B used low-fat (9.0%) raw ground beef; BD, BR, ON and OA used high-fat (26.3%) ground beef.

meat with higher NHI content than BD or BR; percent increase of NHI due to cooking treatment was more than 50% higher for ON and OA compared to BD and BR (Table 3). An extensive heat treatment of the oil-extraction procedure, i.e., cooking meat in oil to ≈105°C, apparently caused increased release of iron from meat pigments. NHI increase in meat upon cooking has been attributed to iron released from myoglobin and hemoglobin (Schricker and Miller, 1983; Chen et al., 1984; Buchowski et al., 1988; Han et al., 1993). Percent decrease of heme iron due to cooking treatment was higher for BR, ON, and OA than for BD. A substantial portion of heat-coagulated hemoproteins (meat pigments) on the surface of meat particles might have been removed by the water rinsing step (BR, ON, and OA).

Fatty acid composition

Cooking the high-fat ground beef with ON or OA caused the most changes in fatty acid profile. ON and OA samples contained less 16:0—the most abundant saturated fatty acid in meats (Rhee, 1992)—than BR and BD samples (2.4 g/100g for ON and OA vs 2.9 and 3.3g/100g for BR and BD, respectively) and more 18:2 (>2.8g/100g for ON and OA vs 0.3g/100g for BR and BD), the major polyunsaturated fatty acid in these samples and meat in general. Consequently, ON and OA substantially increased total polyunsaturated fatty acid (PUFA) content and decreased total saturated fatty acid (SFA) content (Table 4). ON and OA samples, therefore, had the highest PUFA to SFA ratio (>0.8 for ON and OA vs <0.1 for BD and BR). The sharp fatty acid composition change with ON or OA was due to the composition of the cooking oil (soybean oil) used for extraction of meat, i.e., >60% PUFA (mostly 18:2) vs ≈15% SFA (USDA, 1979).

Lipid oxidation

TBA values of cooked samples as related to storage time (days) at 4°C were compared (Fig. 1) for the high-fat meat cooking treatments. TBA values at day 0 to day 2 were lower for ON and OA samples than for BD and BR samples. However, upon extended storage (6 or 8 days), TBA values of ON and OA samples exceeded those of BD and BR samples. Soybean oil used for ON and OA might have contained some natural antioxidants (such as tocopherols), providing a lag period for lipid oxidation. However, with extended storage, this antioxidant effect apparently did not counteract the effects of higher concentrations (in ON and OA samples) of PUFA (Table 4), the fatty acid group most prone to peroxidation, and nonheme iron (Table 3), a major catalyst of lipid oxidation in cooked meat (Rhee, 1988).

Sensory properties

Data from the trained panel evaluation of cooked ground beef samples (with no other ingredients added) indicated that, of all

Fig. 1—TBA values of samples stored at 4°C as affected by cooking method and storage time.

the flavor attributes evaluated, "cooked beef/brothy" (CBB) note, a typical positive note (Johnsen and Civille, 1986), was the only attribute whose intensity scores differed significantly among high-fat meat cooking treatments. BD samples (mean score, 3.93) were perceived more intense in CBB than ON and OA samples (3.47 and 3.40, respectively); BR samples (3.60) were not significantly different than BD samples. ON and OA samples had undergone more extensive extraction/separation processes, i.e., extraction with hot oil followed by rinsing with hot water, and thus had less beefy flavor. Adding back the concentrated meat broth to cooked meat did not restore the flavor lost from the extensive treatment.

When the meat products (meatloaf, spaghetti sauce, and chili) made with ground beef precooked by BD, BR, ON, or OA were evaluated by consumers (a large number of untrained panelists), flavor and overall acceptability scores were significantly affected by precooking treatment for each of the 3 products, whereas texture scores were not (Table 5). Meatloaves prepared with the meat precooked by OA were liked least in flavor; BD, BR, and ON precooking treatments yielded meatloaves with similar (p > 0.05) flavor scores. Meatloaves made with BR-precooked meat received higher overall acceptability scores than those made with OA-precooked meat. For spaghetti sauce, both flavor and overall acceptability scores were higher when made with BD or BR meat than with ON or OA meat. Similarly, BD and BR chili samples received higher flavor scores than ON and OA chili samples. Overall acceptability scores were not significantly different among BD, BR, and OA chili samples; ON samples received the lowest scores. Overall acceptability scores corre-

Table 5—Consumer panel sensory scores for products made with ground beef precooked by different methods

Cooking[c] method	Meatloaf			Spaghetti sauce			Chili		
	Flavor	Texture	Overall accept.	Flavor	Texture	Overall accept.	Flavor	Texture	Overall accept.
BD	4.16[a]	4.14[a]	3.98[ab]	4.96[a]	4.72[a]	5.06[a]	5.17[a]	4.96[a]	5.04[a]
BR	4.68[a]	4.28[a]	4.48[a]	4.88[a]	4.64[a]	4.86[a]	5.12[a]	4.85[a]	5.00[a]
ON	4.26[a]	4.02[a]	4.10[ab]	4.32[b]	4.78[a]	4.46[b]	4.31[b]	4.63[a]	4.37[b]
OA	3.60[b]	3.94[a]	3.68[b]	4.08[b]	4.46[a]	4.16[b]	4.94[b]	4.90[a]	4.88[a]

[a,b] Means within the same column with the same superscript are not significantly different (p>0.05).
[c] BD, BR, ON and OA used high-fat (26.3%) ground beef.

lated highly with flavor scores for each product ($r = 0.85$; $p < 0.001$). When data on all three products were combined for statistical analysis, both flavor and overall acceptability scores were higher for BD and BR samples than for ON and OA samples.

CONCLUSIONS

EXTRACTION of high-fat ground beef by cooking in vegetable oil and rinsing with hot water resulted in lower reduction of fat and cholesterol than browning (with no oil added) and hot-water rinsing. In addition, the oil extraction method, ON or OA, has many disadvantages. It not only requires longer preparation time but also yields less palatable products. When consumers purchase high-fat (or "regular") ground beef, rather than lean or extra lean ground beef, BD or BR cooking technique is recommended, rather than the oil extraction method, to reduce fat and cholesterol.

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Ms received 11/14/94; revised 3/27/95; accepted 4/8/95

Beef Shank Fat Solubility in Supercritical Carbon Dioxide-Propane Mixtures and in Liquid Propane

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ABSTRACT

For propane, conditions were 298K and 308K at the liquid vapor pressure. For supercritical mixtures, conditions were 308K at pressures from 8.9 to 15.7 MPa. The solubility of fat in CO_2-propane increased with increasing pressure, up to 0.66wt% at 15.7 MPa. The average solubility of the fat in propane at 298K was 16.7wt% and at 308K it was 21.4wt%. The extracted fat was solid with a melting range of 295-314K. There was no apparent degradation of collagen during extractions. Beef shank fat extraction with propane is feasible and advantageous over use of supercritical CO_2-propane.

Key Words: beef shank, fat solubility, supercritical CO_2, propane

INTRODUCTION

COLLAGEN is a fibrous polypeptide that contains a variety of amino acids arranged in 3 helically coiled chains. Collagen is the principal component of skin, connective tissues and bones. It is usually obtained from porkskins and to a lesser degree from cattle hides and bones. Collagen has many uses for food, pharmaceutical, biomedical and photographic products. Its main use in the food industry is in manufacture of gelatin and sausage casings. Gelatin is obtained from partial hydrolysis of collagen (Eckroth, 1984; Elvers et al., 1989). Sausage casings are made by extruding an acidic defatted collagen suspension (Boni, 1993).

Collagen for manufacturing sausage casings is obtained from the corium layer split from bovine hides. A possible new, lower cost source of collagen may be a by-product from the process for desinewing meat from beef shank with a Baader "desinewing" machine (Boni, 1993). This beef shank by-product consists of (by wt) ≈28% collagen, 21.5% fat, 50% water and 0.5% protein and inerts. However, the by-product contains a large amount of fat that must be removed before the collagen can be converted into sausage casings. The fat extraction process is limited to temperatures below 313K to avoid collagen degradation.

Industrial processes for removing fat use liquid solvents such as n-hexane or ethyl ether. However, solvent substitutions are being sought because of residual solvent in some products, high solvent recovery costs, and government regulations concerning health and environmental hazards. Possible solvents for fat extraction include supercritical fluids or low molecular weight hydrocarbons (Boni, 1993).

Carbon dioxide (CO_2) has been the most widely used supercritical fluid solvent for removing fats and oils from different materials. Fattori et al. (1988), Eggers and Sievers (1989), Tilly et al. (1990), and Temelli (1992) reported on the extraction and fractionation of seed oils with supercritical CO_2. Chrastil (1982), Bamberger et al. (1988), Brunetti et al. (1989), Gonçalves et al. (1991), Nilsson et al. (1991), Hammam (1992), and Bharath et al. (1992) reported solubility and vapor-liquid equilibria of some pure triglycerides in supercritical CO_2. Typical extraction conditions were temperatures 313 to 353K and pressures up to 35 MPa. Higher temperatures, pressures and amounts of solvent were required to achieve notable solubilities. Even at those conditions, the solubility of fats or oils in supercritical CO_2 was usually <1wt%. Such conditions are far too extreme and solubilities too low to be practical for an industrial beef-shank fat removal process.

The use of entrainers or cosolvents with supercritical CO_2 is a method for increasing solubilities of fats. Ikushima et al. (1985) studied ethyl acetate, ethanol, ether, and acetone as cosolvents, and found that ethyl acetate resulted in higher solubilities of solute in supercritical solvents. Brunner and Peter (1982) evaluated entrainers such as ethanol, acetone, hexane and chloroform. However, such methods use liquid cosolvents which are likely to partition in the solid collagen-water phase and therefore have some of the disadvantages of liquid solvents.

Drew and Hixson (1944) and Bogash and Hixson (1949), measured liquid-liquid equilibria of various triglycerides, long chain fatty acids, and vegetable oils in near-critical propane. Their results led to development of the Solexol process for fractionating vegetable and fish oils with commercial propane. The process was discontinued for more economical liquid extraction processes based on hexane or chlorinated solvents.

There has been renewed interest in propane primarily due to environmental problems with solvents. Peter et al. (1987) used a CO_2-propane mixture for separation of lecithin from mono-, di- and triglycerides in soya oil. Coorens et al. (1988) measured phase equilibria boundaries for tripalmitin with propane. Straver et al. (1993) measured liquid-liquid-vapor boundaries for tripalmitin and tristearin with propane. Hartel and Wang (1993) extracted milk fat with liquid propane.

Some studies have been reported on removal of fats from meat matrices using supercritical CO_2. King et al. (1989) extracted fat from porcine lard, link sausage, smoked ham, and a low-fat ham with supercritical CO_2 at 353K and pressures of 34.5 and 69 MPa. They extracted >96% of the fat after extensive grinding, homogenization and dehydration of samples. Chao et al. (1991), Bailey et al. (1993), and Chao et al. (1993) investigated extraction and fractionation of fats and cholesterol from ground beef meat and beef tallow at 303 to 323K and pressures from 10.3 to 31 MPa. Higher temperatures and pressures were required to obtain acceptable amounts of extracted fat. Bailey et al. (1993) also investigated the extraction of the beef tallow with CO_2 entrained with ethanol.

Our objective was to examine the feasibility of extracting fat from the beef shank by-product using supercritical carbon dioxide with propane as cosolvent and liquid propane. Carbon dioxide is low cost, nontoxic and has a minimum of health hazards. Its greatest limitation is the low solubility of fat at lower temperatures. Propane has an excellent affinity for fats. By combining propane with supercritical CO_2, we expected that the fat solubility would greatly increase. Liquid propane, although flammable, is gas at atmospheric conditions. It was favored for this process because it was more easily recovered and recycled than some liquid solvents.

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MATERIALS & METHODS

PROPANE AND CARBON DIOXIDE of 99.99 mol% minimum purity were obtained from Sunox, Inc. (Columbia, SC) and used without further purification. The CO_2 cylinder was supplied with an educator tube for delivery of liquid. The beef shank material (BSM) was obtained from IBP, Inc. (Dakota City, NE). It had been generated with a Baader desinewing

machine and was derived from bovine connective tissue instead of the corium layer of bovine hides.

The BSM consisted of (by wt) $\approx$28% collagen, 25% fat, 45% water and 2% protein and inerts by hexane/diethyl ether extraction, moisture test and ash content tests. The crude fat had an average saponification value of 192.3 (AOCS method Cd 3-25, Mehlenbacher, 1970) and a melting point range of 295–314K.

Solvent composition analysis

Compositions of CO_2-propane mixtures were determined using a Perkin-Elmer 3920 gas chromatograph equipped with a thermoconductivity detector. A 2mm, 80/100 mesh Hayes Sep D packed column at a temperature of 373K, was used for separation of the gases. Relative weight response between solute concentration and volume were made with pure propane and pure CO_2. Propane content in CO_2 mixtures could be determined ± 1%. Solubilities of fat in both solvent groups were determined by weight difference.

Preparation of material

The BSM was ground once with a 6mm meat grinder prior to extraction. The grinder consisted of a cylindrical die (10 mm thick $\times$ 50 mm diam) with thirty 6 mm holes, with a hand-driven screw feed. The ground BSM was separated into various batches of $\approx$400g each and stored at 277K until use. For extraction, each batch was weighed and mixed with 25mm ceramic berl saddles (Norton Chemical Process Product Corp., Akron, OH) in a 2:1 ratio (BSM/saddles) before loading into the extraction vessel to increase solvent-solute contact area.

Extraction apparatus

The extractor vessel and recirculating pump were contained in a water bath (Fig. 1) that was maintained to ±0.2K with a controller (Omega model CN9000) in proportional mode, and a 100 ohm platinum resistance thermometer. The 1000 mL extraction vessel was made of 316 SS and was rated to 58.6 MPa. A dip tube was machined into the vessel to promote contact between the circulating fluid and the beef shank product. The magnetic pump was a design by Drake et al. (1990) and could circulate liquids at a rate of 200 mL/min. All valves and fittings used in the equilibrium portion of the apparatus were medium pressure, coned and threaded (HIP, Inc., Erie, PA), rated to 68.9 MPa and made of 316 SS.

Liquid CO_2 was filtered and introduced into the extraction vessel through V1, where it contacted the sample. A back pressure regulator and a relief valve (Nupro R3A series, cracking pressure of 41.36 MPa) were incorporated in the apparatus for safety. Propane was introduced into the apparatus through V3. For the extractions with CO_2-propane, a 250 mm long, 14.3 mm O.D. high pressure nipple filled with silica gel was connected between V8 and a micrometering valve, V9. The system could be depressurized with V9. The collector was used to check for unabsorbed fat. Solvent gas volume could be measured with a wet test meter. For extractions with propane, a sampling arrangement consisting of a 75 mL stainless steel sampling bomb and several valves were connected to V5 (Fig. 1). By manipulating valves V2-V5, V8 and V9, samples of the supercritical phase or of a liquid phase could be taken for analysis.

Temperature of the water bath was measured by a mercury thermometer with a certified N.I.S.T. traceable accuracy of ±0.05K. The temperature cycled ±0.2K slowly over a 5 hr period. Pressure of the extraction vessel was measured with a transducer (Ashcroft model ASHK1, Dresser Industries, Stratford, CT) of nominal 0.5% accuracy. The pressure readout (Ashcroft model 2269A) displayed pressure from 0 to 34.5 MPa to the nearest 0.069 MPa.

Experimental procedure

Extraction with CO_2-propane mixture. The BSM mixed with the berl saddles was introduced into the extraction vessel. The apparatus was then purged several times with gaseous CO_2 up to 5.51 MPa at room temperature until only CO_2 was present as determined by GC. The water bath was then cooled to 276–278K with a submersible cooler. To load the propane, V2 and V7 were closed with V4 and V6 open. The loop between V4 and V7 was then filled several times with propane by opening V3. After each filling, V7 was opened to allow propane to flow into the system. At the end of this procedure, the pressure in the vessel was 0.55 MPa. The vessel was then filled with liquid CO_2 until the pressure approximately equilibrated with that of the CO_2 cylinder. The bath was

heated to the extraction temperature and equilibrated within 2 hr. To saturate the solvent with the fat, the liquid phase was recirculated through the vessel by opening V2, V4 and V7, and the pump was started. The system was allowed to saturate for 45 min. The initial pressure in the vessel was recorded. With V8 opened, the micrometering valve was slowly opened and gas allowed to flow through the silica gel, the water trap and into the wet test meter. V8 was then closed and after no more gas exited, the volume of gas was recorded and the packed nipple was taken off-line and reweighed. The difference in weights gave the amount of fat extracted. In each run, about the same volume of CO_2 was allowed to slowly flow through the silica gel. Above 11.03 MPa, the pressure drop was $\approx$1.45 MPa, below 11.03 MPa, it was $\approx$0.34 MPa. Our objective was to determine the feasibility of the solvent mixture and this procedure was judged to be adequate.

Extractions with the CO_2-propane mixtures were performed with the same batch of material at 308K. Most previous studies indicate that solubilities increased with increasing temperature. Therefore, the highest temperature that would cause no collagen degradation was used. Each run consisted of repeating the above procedure for a given batch of BSM.

Extraction with propane. After introducing the BSM mixed with berl saddles into the extraction vessel, the apparatus was purged with gaseous propane at 0.5 MPa. The water bath was cooled to 283–287K and propane was loaded for 30 min to ensure sufficient liquid inside the vessel. It was then heated to extraction temperature and equilibrated within 2 hr. The liquid solvent was recirculated through the vessel with V2, V4, V6 and V7 opened, V3 and V5 closed, and starting the magnetic pump. After 30 min, the magnetic pump was stopped. This saturation time was judged adequate from bench lab test results using liquid solvents.

With V6 and V7 closed, a sample of liquid was slowly taken into the preweighed sampling bomb through V5. Because the system consisted of a vapor-liquid mixture, the pressure and temperature could be held constant as a small amount (30g) of liquid was withdrawn. The bomb was reweighed to determine the total weight of liquid propane and extract, and then connected to the micrometering valve V9. The bomb was depressurized by opening VC and V9 slowly. This allowed propane gas to flow through the micrometering valve, bubble through a water trap and into the wet test meter. After no more gas exited, the bomb was reweighed to determine the amount of extract. This depressurization required between 1.5 hr at 1.04 MPa, and 2.5–3.5 hr at 1.31 MPa to prevent fat entrainment. The sample bomb was cleaned, weighed, and the sampling arrangement reconnected to the system. A second extraction was made at the same conditions using the same procedure.

Six different batches of BSM were used for propane extractions. Three batches were extracted at 298K and three batches extracted at 308K. Two extractions were made with each batch which consisted of different samples of BSM.

RESULTS & DISCUSSION

Material extracted

The material extracted with the CO_2-propane mixture and adsorbed onto silica gel was yellow. This was apparent because the silica gel was initially white. During preliminary and final runs, the collector was monitored for unadsorbed fat. Only weight and visual analyses were made.

The material extracted with propane was collected in a sample bomb. Immediately after opening the bomb, the material extracted was brownish and foamy, with large bubbles. After several minutes, the bubbles were much smaller. This foamy appearance was probably due to the mixing effect between fat, water and propane during depressurization. When heated to 403K, samples were an intense yellow colored oil. After cooling to 295K, samples were semi-solid, a mixture of yellow liquid and white solid. This semi-solid appearance indicated the presence of saturated and unsaturated fats. The material consisted of about 98.5wt% fat, which was determined by drying extracted material at 403K for several hours. The fat had a melting point range of 295–314K. No differences in melting point were found between fat extracted at 298K and that at 308K. Solubilities reported were corrected for water content.

Solubility of beef shank fat in CO_2-propane mixtures

The first solvent group to be tested for feasibility to extract BSM fat was a CO_2-propane mixture. The temperature used was
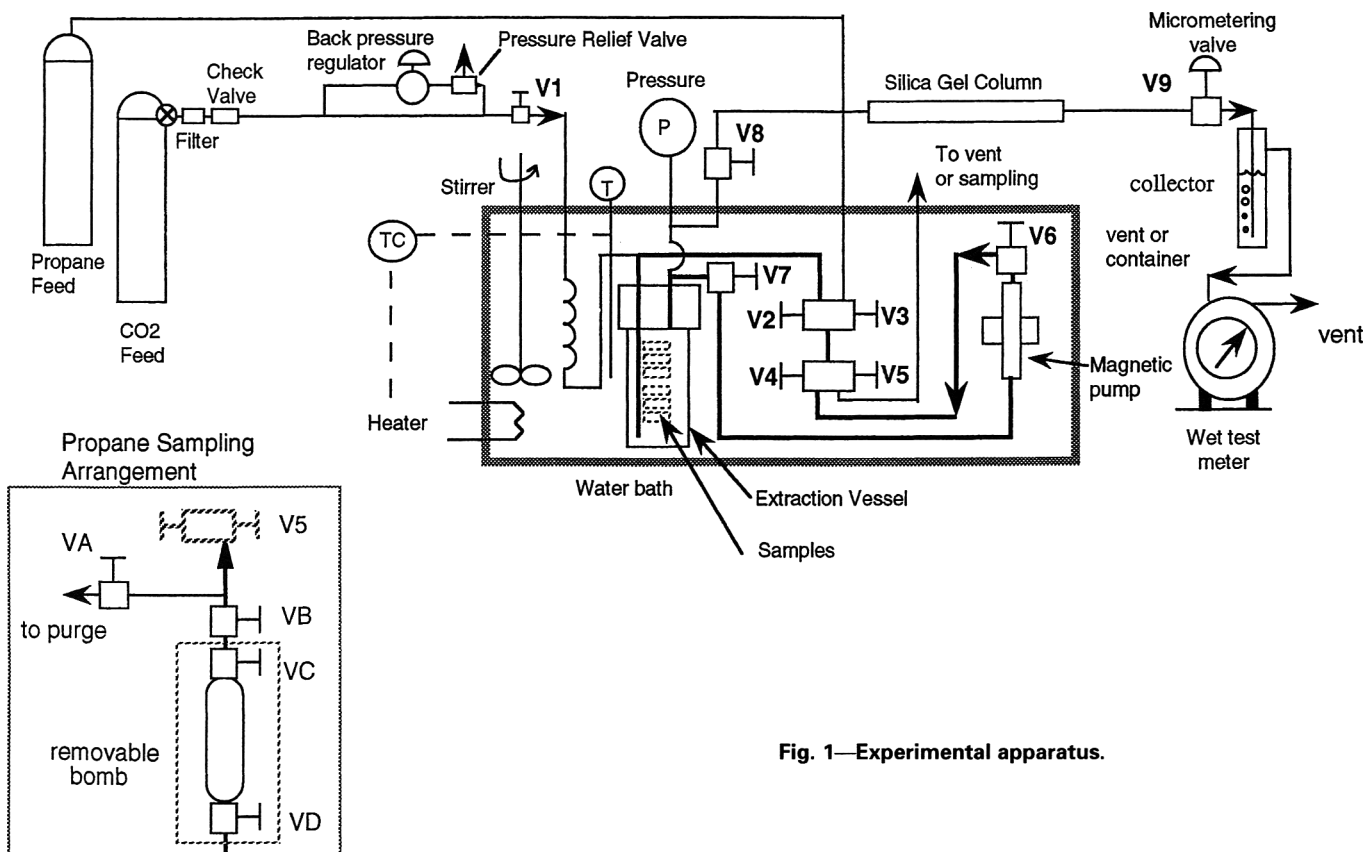
Fig. 1—Experimental apparatus.

308K and pressure ranged from 8.92 MPa to 15.72 MPa. Solubility of the BSM fat in the CO_2-propane mixtures was measured as a function of pressure (Fig. 2). Since concentration of propane could not be fixed exactly at a given value, each run had a slightly different propane concentration. Extractions with 7wt% and 8wt% propane were compared (Fig. 2) as well as solubilities of beef tallow, ground beef fat, tripalmitin, triolein and olive oil at similar conditions.

Solubility of the fat in supercritical CO_2-propane did not vary much between propane at 7 and 8%. There was some scatter in data, but the solubility of fat in both extractions increased with increasing pressure. Solubility increased gradually (0.03%/MPa) as pressure was increased between 9 MPa and 14 MPa. Between 14 MPa and 16 MPa, solubility was more dependent upon change in pressure (0.2wt%/MPa).

The solubility of fat in the CO_2-propane mixture was expected to be close to the concentration of propane in the mixture, that is, 7wt%. Bogash and Hixson (1949) reported complete miscibility of triglycerides in liquid propane above the melting point of the triglycerides. Therefore, the amount of propane in the solvent could presumably dissolve an equal amount of fat. Since at our conditions the fat was not above its melting point, the solubility of the fat would be lower than the amount of propane in the solvent (7wt%). However, considering that the mixture was at supercritical conditions (Hicks and Young, 1975), the solubility could be enhanced to near 7wt%. The solubility was <0.65wt% at pressures below 15 MPa. Part of this lower solubility could be due to the presence of water in the BSM.

A comparison of BSM fat solubility data with extraction studies of beef tallow, ground beef fat, tripalmitin, triolein and olive oil was made (Table 1). Solubilities of fats and oils in CO_2-propane mixtures were not available. Therefore, only solubilities in pure CO_2 or in mixtures of CO_2 and other substances are shown. Furthermore, most of the data were above 313K and 17.23 MPa which are more severe conditions than those of interest. The solubility of the BSM fat was expected to behave similarly to that of ground beef and beef tallow in pure CO_2.
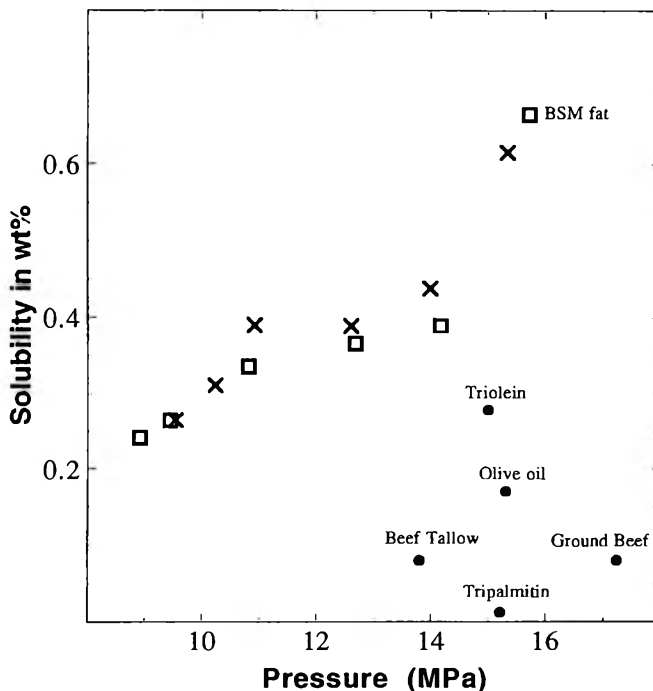
Fig. 2—□ Solubility of BSM fat in CO_2 + 7wt% propane at 308 K; X Solubility of BSM fat in CO_2 + 8wt% propane at 308K; ● Solubilities of other fats in CO_2. Sources and conditions are given in Table 1.

The solubility trend was also expected to be comparable to that of other fats and oils.

The presence of propane appeared to have a considerable enhancement effect, especially considering the mild conditions. The solubility of ground beef at 308K and 17.2 MPa as measured by Chao et al. (1991), was ≈0.08wt% in pure CO_2. For

Table 1—Comparison of solubilities of fat in supercritical CO_2

Substance	Solvent	Temperature (K)	Pressure (MPa)	Solubility (wt%)
Beef shank	CO_2 + 7wt% propane	308	15.7	0.66
Ground beef[a,b]	CO_2	308	17.2	0.08
Ground beef[a,b]	CO_2	308	31.0	0.14
Beef tallow[a,c]	CO_2	313	13.8	0.08
Beef tallow[a,c]	CO_2 + 5wt% ethanol	313	13.8	0.32
Beef tallow[a,c]	CO_2	313	24.2	0.43
Beef tallow[a,c]	CO_2	313	34.5	0.90
Triolein[d]	CO_2	308	15.0	0.28
Olive oil[d]	CO_2	308	15.3	0.17
Tripalmitin[e]	CO_2	313	15.2	0.013

[a] Average solubility estimatec from the referenced extraction data.
[b] Chao et al. (1991).
[c] Bailey et al. (1993).
[d] Gonçalves et al. (1991).
[e] Bamberger et al. (1988).

Table 2—Solubility of BSM fat in liquid propane

Temp (K)	Pressure (MPa)	No. extractions	Avg solubility (wt%)	Standard deviation	95% Confidence intervals
298	1.04	6	16.7	0.60	(15.1-18.2)
308	1.31	6	21.4	0.43	(20.3-22.5)

Table 3—Solubility of BSM fat in propane and experimental design

Extraction	Temp (K)	Pressure (MPa)	Solubility (wt%) Extraction 1	Solubility (wt%) Extraction 2
1	308	1.31	21.36	21.38
2	298	1.04	17.47	16.58
3	298	1.04	15.65	16.72
4	308	1.31	20.77	21.85
5	298	1.04	16.78	17.00
6	308	1.31	21.91	21.16

beef tallow at 313K and 13.8 MPa, Bailey et al. (1993) also obtained a solubility of 0.08wt%. The solubility of the beef tallow increased to 0.32wt% when the CO_2 was entrained with 5wt% ethanol at the same conditions. The effect of propane on solubility enhancement appeared to be similar to that of ethanol. That is, the solubility of BSM fat would have been lower in pure CO_2 at the same conditions.

The solubility could be increased by increasing the pressure. Chao et al. (1991) and Bailey et al. (1993) investigated the effects of pressure on extraction of ground beef and beef tallow, respectively, with pure CO_2. The solubility of ground beef fat at 308K and 17.2 MPa was 0.08wt%. Increasing the pressure to 31 MPa increased the solubility to about 0.14wt% at the same temperature. For the beef tallow, the solubility increased from 0.08wt% at 313K and 13.8 MPa, to 0.43wt% at 24.2 MPa, and up to 0.9wt% at 34.5 MPa. This corresponded to an increase of 11% in solubility from 13.8 to 34.5 MPa. If the BSM fat followed the same trend, increasing the pressure to around 34.5 MPa might increase the solubility to 7wt%. However, several oils exhibited maximum solubilities at a given pressure (Stahl et al., 1988). Above that pressure, solubility started to decrease. Pressures >15.72 MPa were not investigated. Higher temperatures could not be used to increase solubilities because of collagen degradation. Higher concentrations of the entrainer could lead to higher solubilities. However, the entrainer concentration was limited to the mixture critical point of CO_2-propane at 308.2K which is 17wt% (Hicks and Young, 1975). Above that propane concentration. a liquid propane phase would develop.

The solubility dependence on melting point of the triglycerides was discussed by Hammam (1992, 1994). Tripalmitin, a saturated triglyceride, solid at the conditions reported, had the lowest solubility. Triolein, an unsaturated triglyceride, is a liquid and therefore, exhibited a higher solubility than tripalmitin. Refined olive oil, also a liquid, is a mixture of triglycerides. Its major constituent is triolein but it also contains triglycerides

with fatty acid chains of lower solubility than oleic acid. This probably explains its lower solubility compared to that of pure triolein (Gonçalves et al., 1991).

The solubility of the BSM fat in the CO_2-propane mixtures was the same order of magnitude as the solubility of other fats and oils in supercritical CO_2. The BSM fat, which has a melting point range intermediate to tripalmitin and triolein, was expected to have solubilities intermediate to these fats and oils in pure supercritical CO_2, as with the beef tallow and ground beef fat. The fact that BSM fat had higher solubilities confirmed the enhancement effect of the propane on solubility.

Solubility of beef shank fat in liquid propane

A total of six extractions of BSM with liquid propane were made at two different temperatures. Three batches of BSM were extracted at 298K and three were extracted at 308K. Two consecutive extractions were made with each batch. The average solubilities of the BSM fat in liquid propane at 298K and 308K (Table 2) were determined from statistical analysis of experimental data. Solubility increased with increasing temperature. It was 28% higher at 308K than at 298K and higher solubilities were expected at 308K. Bogash and Hixson (1949) reported pure liquid triglycerides were totally miscible with propane. The system temperature was very close to the upper melting point of the BSM fat, thus higher solubilities were expected.

The solubility data (Table 2) could be interpolated by using the standard thermodynamic equation for solid-liquid equilibria:

$$\ln W = -\Delta H^{fus}/RT + (\Delta H^{fus}/RT_m - \ln(\gamma)_{avg})$$

where W is the weight % of the fat, ΔH^{fus} is the heat of fusion, T is temperature in K, T_m is the melting temperature of the fat, and $(\gamma)_{avg}$ is an average activity coefficient. For these data, T_m = 313.15K, $\Delta H^{fus}/R$ = 2276 K, and ln $(\gamma)_{avg}$ = -3.1849.

Statistical analysis results

The statistical experimental design (Table 3) and the solubilities of the BSM fat in propane for all extractions were compared. Each one represents a different sample of BSM, therefore, three replicates were made at each temperature. The extractions were randomized with temperature. An analysis of variance (ANOVA) was performed with two factors, temperature (298, 308K) and extraction (1 and 2) with the Statistical Analysis System (SAS Institute, Inc., 1993) software package. Extractions 1 and 2 represented the two consecutive extractions. In all analyses significance was established at p ≤ 0.05.

Analysis of normality and residuals showed that the data followed a normal distribution. Analysis of the full model indicated that the interaction between temperature and extraction was not significant (p = 0.98) and therefore, this term was dropped from the model. Analysis of the reduced model indicated that the effect of the extraction was not significant (p = 0.70). This meant that in both consecutive extractions, the sample of propane containing the solubilized fat was taken at equilibrium conditions. There was no evidence that consecutive solubilities were different. The effects of temperature were significant (p = 0.0001) with solubility increasing with increasing temperature. As a result of analysis, 6 points were used to calculate average solubility at each temperature.

The residual fibrous material after extraction with both solvent groups was pleasant smelling, with no indication of rancidity or decomposition of collagen material. The propane extracted material was smooth and much leaner than the original material.

Comparison of extractions between solvents

At 308K, the solubility of fat in propane was almost 33 times more than that in the CO_2 propane mixtures. Estimate calculations were made to determine the implication of such data on ultimate extraction, process design, and economics. Solubility data are time independent whereas continuous extraction data are time dependent. From solubility data, one may use crosscurrent or countercurrent leaching equations to estimate the number of contact stages that would be required to lower the fat content in the BSM to the desired level. This would be difficult to estimate from continuous extraction data due to its time dependent nature.

The target fat content of the BSM is 5wt% of solids content. Assuming countercurrent extraction and constant distribution coefficients, the number of required stages for each solvent could be calculated with standard techniques (Henley and Seader, 1981). For propane, a minimum of four contact stages and solvent to feed ratio (S/F) of two would be required. For CO_2-propane, 13 stages and a S/F of 40 would be required. Economic calculations show that the cost with liquid propane for processing 2.3 million kg/year of BSM material on a 330 day, 24 hr basis would be $\approx$ four times lower than that if CO_2-propane mixtures were used.

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Ms received 11/22/94; revised 3/4/95; accepted 4/10/95.

Reversible pH Effect on Pork Paleness in a Model System

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ABSTRACT

Pork was cut into small disks, perfused with phosphate buffer, and fiber-optic reflectance was measured as pH was changed under computer control. After myoglobin removal, the effect of pH on paleness was reversible. From pH 7 to pH 5, reflectance increased as pH decreased, and decreased as pH increased (P < 0.005). A notable degree ($\approx$40% total reflectance) of light scattering from normal pork may originate from a reversible effect of pH on myofibrils. However, this makes only a minor contribution ($\approx$5% total reflectance) to the very high reflectance of severely pale, soft, exudative pork.

Key Words: pork, fiber optics, reflectance, pH, paleness

INTRODUCTION

THREE HYPOTHESES have been proposed to explain how low pH causes paleness in PSE (pale, soft, exudative) pork. Firstly, Hamm (1960) proposed that shrinkage of myofibrils at a low pH increased the refractive index difference relative to surrounding sarcoplasm, so that reflectance at the myofibrillar surface was increased. Supporting evidence came from scanning confocal light microscopy (Offer et al., 1989). Secondly, Bendall (1962) and Bendall and Wismer-Pedersen (1962) found by light microscopy that sarcoplasmic proteins were precipitated in PSE pork. Porcine myoglobin is more susceptible to acid denaturation than are bovine and ovine myoglobins (Satterlee and Zachariah, 1972) and also there is evidence of myosin denaturation in PSE pork (Stabursvik et al., 1984). Thirdly, an increased myofibrillar refractive index at low pH may increase the angle of refraction of light passing through myofibrils. Supporting evidence came from birefringence measurements by ellipsometry (Swatland, 1989), coupled with single-fiber transmittance measurements (Swatland, 1990).

The relative applicability of these three hypotheses (reflection, precipitation, and refraction) is unknown because they are difficult to investigate in naturally occurring PSE and DFD (dark, firm, dry) pork. For example, it seems reasonable that light scattering from protein denaturation and precipitation might predominate in severe PSE pork and that absence of normal reflective and refractive scattering might account for DFD meat, but this has never been tested experimentally.

The objective of my study was to determine if pH-related paleness in pork could be reversed if pH were artificially increased.

MATERIALS & METHODS

Pork samples

Samples were taken from longissimus dorsi, adductor, gluteus medius, or transversus abdominis muscles of typical commercial pork carcasses (n = 15, cross-bred barrows and gilts with hot carcass weight $\approx$ 85 kg) at various times postmortem. The muscles were chosen to give a range from low (longissimus dorsi) to high (transversus abdominis) myoglobin concentration. Starting samples were judged visually for PSE or DFD using the Japanese pork color scale (Nakai et al., 1975).

Sample chamber

Pork samples were trimmed to a disk shape (diameter 10 mm, depth 3 mm). The orientation of the longitudinal axes of muscle fibers either was parallel to the flat face of the disk, or was perpendicular to the flat face. Disks of pork were installed in the sample chamber beneath a stainless-steel screen (square weave, 0.38 mm openings, #40 mesh; Fig. 1, S). The sample disk was packed around the supply tube (outer diameter = 1.5 mm) so that fluid was forced to pass through the sample disk as it moved from the lower to the upper chamber. Fluid (Fig. 1, F) was supplied to the lower surface of the sample disk from a gravity-feed reservoir (30 cm drop), and withdrawn from the upper surface by a negative pressure to give a flow rate of 5 mL min^{-1}. The total volume of the sample chamber was 0.5 mL, hence, fluid was changed about 10 times/min.

The perfusing fluid was 0.2M phosphate buffer, with the pH set by computer (Swatland, 1994). Myoglobin absorption spectra in phosphate buffer were described by Kiese and Kaeske (1942). The absorption spectrum of metmyoglobin is particularly sensitive to pH (Bowen, 1949). Fluid reservoirs for buffer stock solutions and a mixing sump were located on the floor, and fluid was pumped to the gravity-feed reservoir where the pH electrode was located (gel-filled combination electrode, Fisher Scientific model 119). The height of the gravity feed was used to regulate fluid delivery rate to the sample chamber, and a stainless steel mesh (square weave, 0.38 mm openings, #40 mesh) was located on the base of the feed tank to remove bubbles. A large diameter (inner diameter = 7 mm) tube from the feed tank back to the sump (by-passing the sample chamber) reduced pressure fluctuations in the sample chamber caused by the pump, as well as reducing cycling times and increasing fluid mixing.

The pH was regulated by computer using solenoid valves in the supply lines to the pump intake, so that pump stroke volume was the major determinant of the volume of fluid admitted per second of valve open time (0.16 sec mL^{-1}). The relative heights of reservoir valves above the sump fluid level ($\approx$15 cm) and tube diameters (inner diameter = 4 mm) were balanced so that hydrostatic pressure in the reservoir tanks had a minimal effect on the time:volume relationship of valves, or back-flow into the sump between pump cycles. Excess fluid (>100 mL) was drained off through an overflow.

To change the pH, a slight underestimate of the volume of stock solution to be replaced in the buffer was calculated from

$$pH = pK_{a2} + \log[(HPO_4^{2-})/(H_2PO_4^-)]$$

The final pH adjustment was made by an iterative procedure, using learning factors for valve opening times to accommodate changes in hydrostatic pressure in the stock reservoirs. For example, if a valve opening time failed to achieve the expected result, the learning factor was increased to lengthen the opening time next time it was used.

To check that experimental effects were not caused by refractive index changes in the buffer, a continuous flow cell Abbe refractometer (model A, Carl Zeiss, Oberkochen, Germany) was placed in series with the large diameter tube from the feed tank to the mixing chamber. The refractive index of phosphate buffer was $\approx$1.3343, changing by relatively small amounts (0.0002) from pH 5 to pH 7. From studies on the internal reflectance of the junction between optical fibers and aqueous fluids (Swatland, 1991), I considered that pH-related changes in reflectance were not attributable to such small changes in refractive index of the buffer. Furthermore, the apparatus was tested on a sample disk composed of 8 layers of Whatman #1 filter paper, and no significant effects of pH on reflectance were detected (P < 0.05).

Optical system

A 100W halogen light source (Fig. 2, 1) was operated at 12 v 7.825 a from a type 6642A stabilized power supply (Hewlett-Packard, Palo Alto, CA) and was focussed into one branch of a bifurcated light guide through a solenoid-activated iris shutter (Fig. 2, 2; type 467225, Carl
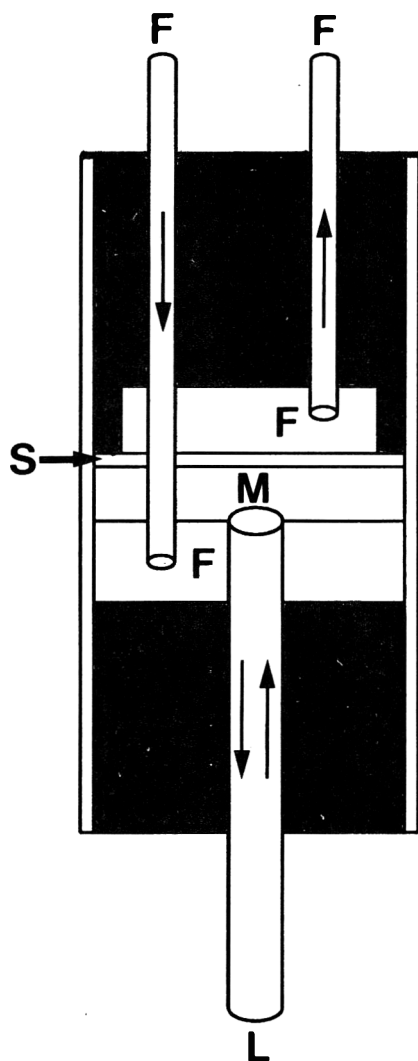
Fig. 1—Diagram of sample chamber, showing stainless-steel screen (S), fluid space (F), meat sample (M), and fiber-optic light guide (L).

Fig. 2—Optical components, comprising halogen source (1), solenoid shutter (2), grating monochromator (3), stray-light filters (4), and photomultiplier (5).

Zeiss, Oberkochen, Germany). The light-guide (type WW100, Guided-Wave, El Dorado Hills, CA) had one branch with six optical fibers (connected to the light source), while the other branch (connected to the photometer) had one fiber. In the common trunk, the six illuminating fibers were arranged in a ring closely around the single recording fiber. The light returned to the recording fiber by reflection and scattering from the sample passed through a grating monochromator (Fig. 2, 3; Zeiss 474321 with 474346 grating), through a stray light filter (Fig. 2, 4; Zeiss 477215) to remove higher-order harmonics, and onto a side-window photomultiplier (Fig. 2, 5; Hamamatsu type HTV R 928, 1126 Ichino-Cho, Hamamatsu City, Japan) with S-20 characteristics.

There is some uncertainty relating to the appropriate name for internal reflectance collected directly from a tissue by optical fibers. The term interactance has been proposed (Conway et al., 1984) but has not been widely accepted. For simplicity, the light collected (sterance) from the sample disks in my study was called "reflectance", although it is not the same as reflectance from the sample surface in air as measured under CIE (International Commission on Illumination) conditions.

The system was operated through an IEEE-488 bus from a Hewlett-Packard 360 microcomputer programmed in BASIC using a Zeiss MPC controller. With the monochromator at 555 nm, the high voltage and gain of the photomultiplier were set to use 80% of its dynamic range. The shutter in front of the illuminator was used to correct for both ambient illumination (which was very low) and the dark current of the photomultiplier (which was subtracted from all measurements). The reflectance standard was a white opal glass disk under distilled water, using an adjustable clamp to find the point just above the glass surface that gave the highest return of light to the photometer. The monochromator was scanned from 400 to 700 nm in increments of 10 nm with a band-pass of 10 nm to find the reflectance maximum at each wavelength.
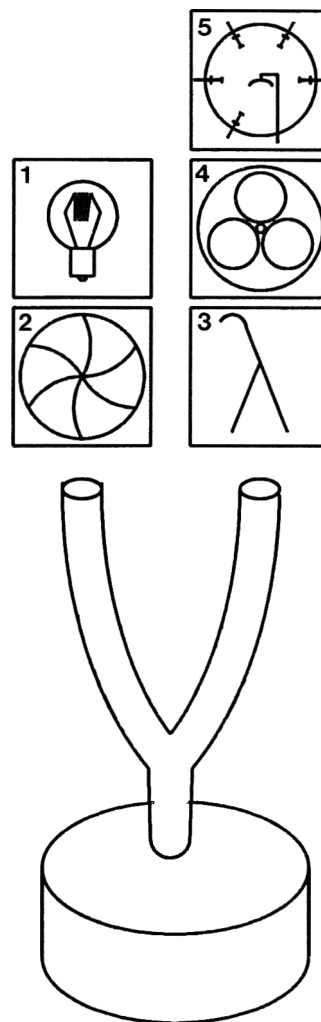
Measurements at static pH

This preliminary study was undertaken without using the computer-controlled apparatus. The foremost technical problem in using a continuously flushed sample chamber under computer control is that reflectance changes caused by the progressive removal of sarcoplasmic chromophores such as myoglobin are superimposed on any effects that might be caused experimentally by changing pH. Thus, the objective of measurements at a static pH was to test that reflectance could be changed by pH change under conditions where myoglobin loss was almost constant (i.e., without continuous perfusion in the computer-controlled apparatus). Fascicular subunits of transversus abdominis were removed from 3 pork carcasses 45 min postmortem and cut into disks along the length of each fasciculus so that the same muscle fibers occurred in each disk. Thus, sampling error from variation in myoglobin content between different histochemical fiber types (Morita et al., 1969) was minimal. Muscle fibers were parallel to the flat face of the disk. At pH 5, 5.5, 6, 6.5 and 7, a muscle disk was placed into 10 mL phosphate buffer for 1 hr at 24°C with slight agitation to keep the myoglobin coloration of the buffer uniform. Samples were measured as if they had been in the sample chamber, but were not placed in the apparatus or perfused.

Measurements at programmed pH changes

The objective here was to separate reflectance changes caused by pH from those caused by myoglobin loss in the continuous-flow cell (Fig. 1), comparing results with those obtained statically. Muscle disks were obtained from the same three pork carcasses as used for static pH measurements, plus multiple samples from all other carcasses at various times postmortem.
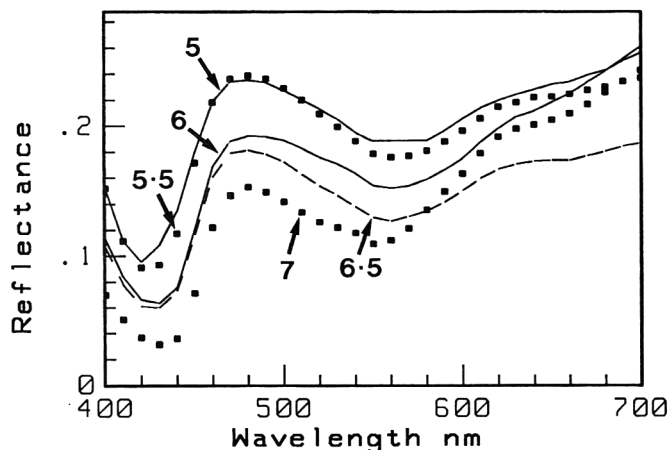
Fig. 3—Reflectance at different static pH values (5 to 7) in separate disks of transversus abdominis muscle with a relatively high myoglobin content. Measurements were made with the light guide gently touching the samples.
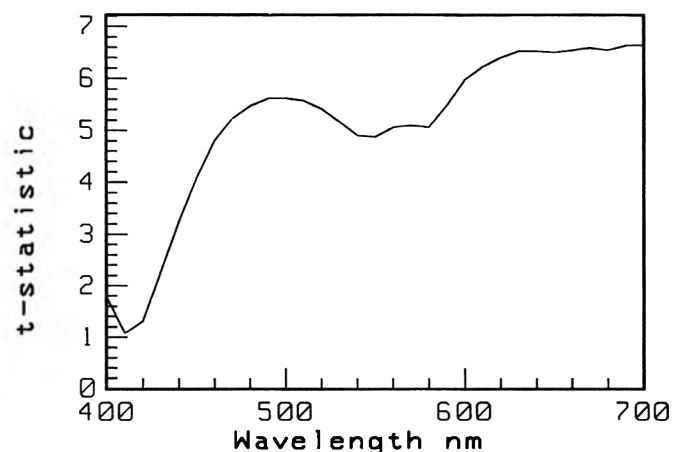
Fig. 4—The t-statistic for the separation of 10 spectra collected at static pH 5.0 from 10 spectra at static pH 7.0. Measurements made with the light guide pushed progressively farther into the samples.

Statistics

Two strategies were used to test the results of pH change. First, when comparing two pH values, a set of 10 spectra at the first pH was compared with a set of 10 spectra at the second pH using a t-test at each wavelength. Second, when evaluating the effect of a progressive change in pH, linear regressions and correlation coefficients were calculated with reflectance on the y-axis and pH on the x-axis, treating each wavelength separately. Software was programmed in BASIC from methods given by Steel and Torrie (1960).

RESULTS

Measurements at static pH

The reflectance of separate disks of transversus abdominis at different static pH values was compared (Fig. 3). The same general effects were observed for all three carcasses. Results from the first carcass are shown as an example. Results were not averaged for the three carcasses because my hypothesis was that variation between disks is a major problem, which is why continuous perfusion of one disk is more sensitive to the experimental effect under examination.

Overall reflectance tended to be higher at low pH than at high pH, but there were irregularities in the spacing of intermediate pH values and in the shape of spectra. For example (Fig. 3), the spectra for samples at pH 6 and 7 deviated from the general
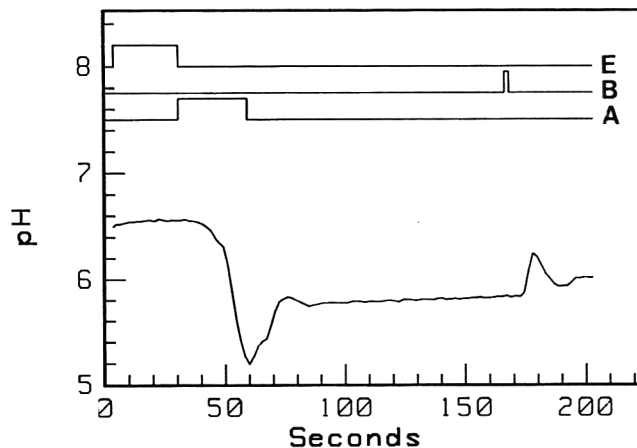
Fig. 5—Example of a programmed pH change from pH 6.5 to pH 6 with respect to time (sec) from the start of a programmed pH change. The status of the exit (E), base (B) and acid (A) valves is shown by arbitrary square waves (valve open when line high).

inverse relationship between pH and reflectance by showing high reflectance at 700 nm. Thus, the inverse correlation of pH with reflectance was confirmed in this data set from 400 to 550 nm ($r = -0.99$, $P < 0.005$ at 550 nm), but not at 700 nm ($r = -0.41$, $P > 0.05$).

Replicate measurements (n = 10) were made at pH 5 and 7 by progressively plunging the probe farther into the sample. This proved the correlation of pH with reflectance at 700 nm, as shown by the t-statistic (Fig. 4). However, experimental error occurred at lower wavelengths because of differences in myoglobin concentration at different depths within the disk, compounded with oxymyoglobin formation on the surface of the disk. The low point of the separation (P > 0.05) was at 410 nm in the Soret absorbance band for myoglobin, with minor low points at 540 and 580 nm, corresponding to the coexistence of both myoglobin and oxymyoglobin. The start of an oxymyoglobin spectrum also may be seen by the flat region between 550 and 580 nm in the spectrum for the sample at pH 5.0 (Fig. 3). This is comparable to the change in surface reflectance when pork is exposed to the atmosphere (Millar et al., 1994). Thus, as the probe was plunged farther into the disk, it contacted parts of the disk with higher residual levels of myoglobin.

Thus, using separate disks, each exposed to a different pH, elevation of pH caused a decrease in reflectance. However, experimental error made this an unreliable method to measure the effect of pH on the reflectance spectrum.

Measurements at programmed pH changes

I used a single disk exposed to a programmed sequence of pH changes but I recognize the importance of the dynamic nature of the pH change (Fig. 5). The system was controlled from three solenoid-operated valves. An exit valve (status shown by Fig. 5, E; valve open when line was high) was used to pump fluid from the system to be replaced by a calculated amount of stock buffer to reach the desired pH. The pH was changed from pH 6.5 to pH 6 (Fig. 5), by replacing the lost fluid with KH_2PO_4 solution through the acid valve (Fig. 5, A), but an overshoot required a small amount of Na_2HPO_4 to be added through the base valve (Fig. 5, B) to reach the target pH. Presumably, integration of these surges in pH took place within the sample chamber or in the outermost layers of the sample disk, because the optical properties of sample disks responded uniformly to changes in pH.

I had to allow sufficient time to remove myoglobin from the disks (at ≈ 1 mm hr^{-1} from each flat face of the disk, so the 3-mm disks reached equilibrium at ≈ 1.5 hr). Thus, a reversible, inverse relationship between pH and reflectance was found in
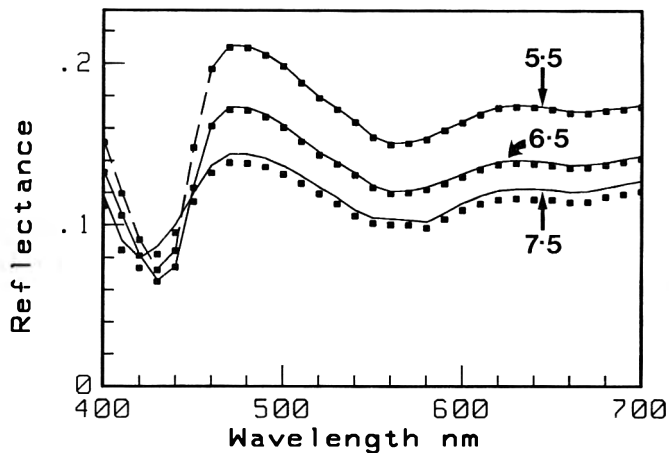
Fig. 6—Reflectance spectra from a disk of porcine transversus abdominis taken 1 hr postmortem, equilibrated to pH 7.5, then programmed to pH 6.5 and pH 5.5 with 10 replicates at each pH. For each pH, the line is the mean value and the squares indicate one SD subtracted from the mean.

Fig. 7—Continuation of changes shown in Fig. 6, with pH programmed back to pH 7.0. For each pH, the line is the mean value and the squares indicate one SD subtracted from the mean.

all 20 tests of the experimental hypothesis in a variety of disks from high to low myoglobin content, from pH 5 to 7, from 45 min to 6 days postmortem, and with muscle fibers either perpendicular to, or parallel with the face. Reflectance decreased when pH was increased, and reflectance increased when pH was decreased.

Problems arose if myoglobin was not washed out of the sample disk adequately (Fig. 6). Proceeding directly to making measurements without first washing out the myoglobin, a disk of transversus abdominis (with relatively high myoglobin) taken 1 hr postmortem was equilibrated for 10 min to pH 7.5, then taken to pH 6.5 and pH 5.5 with 10 replicates at each pH. The effect of pH was obscured in the Soret absorbance band. Thus, from 400 to 410 nm and from 450 to 700 nm the reflectance increased as pH decreased (P < 0.005), while at 430 to 440 nm the effect was obscured.

When the pH was increased, reflectance was decreased (P < 0.005), but not as low as the starting level. For example, the same sample as represented in Fig. 6 was taken to pH 7.0 (Fig. 7), but did not re-attain the relatively low reflectance it originally had at pH 6.5. This was a common observation in all samples tested.

The pH reversibility of light scattering in severe PSE pork (color score < 1) was detectable as late as 6 days postmortem (Fig. 8). The features were typical of a sample with substantial removal of myoglobin: reflectance at the Soret absorbance band was only slightly less than at 700 nm, and spectra at different pH values were parallel. Except at 410 nm in the Soret absorbance band (Fig. 8), reflectance decreased as pH was increased from pH 5 to pH 7, and increased as pH was decreased back to pH 5 (P < 0.005). The effect was detectable with both muscle fiber orientations within the disk.

DISCUSSION

MEASUREMENTS of pH and paleness have proved valuable for investigating the epidemiology of PSE. Meat scientists in many countries have accumulated a broad data base (Bendall and Swatland, 1988). Great progress has been made in elucidating how genetic changes in Ca^{++}-release channels of the sarcoplasmic reticulum may trigger rapid glycolysis and the formation of PSE meat (MacLennan and Phillips, 1992). However, there are indications that PSE may have only a 20% heritability in some situations (de Vries et al., 1994). Its minimization is the responsibility of abattoir operators as well as pig breeders. Thus, new methods are needed to investigate the mechanisms by which it develops.
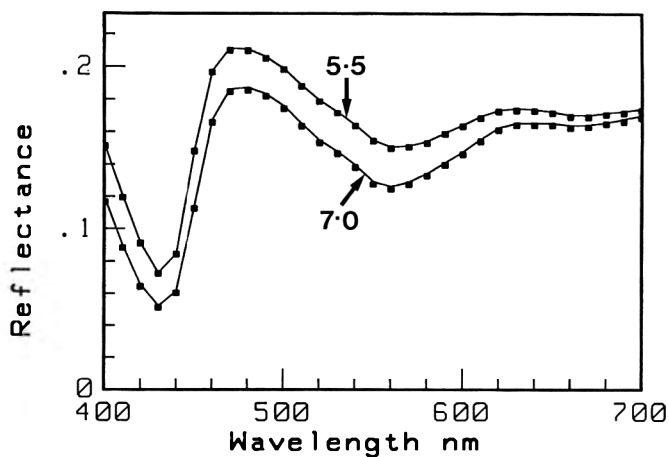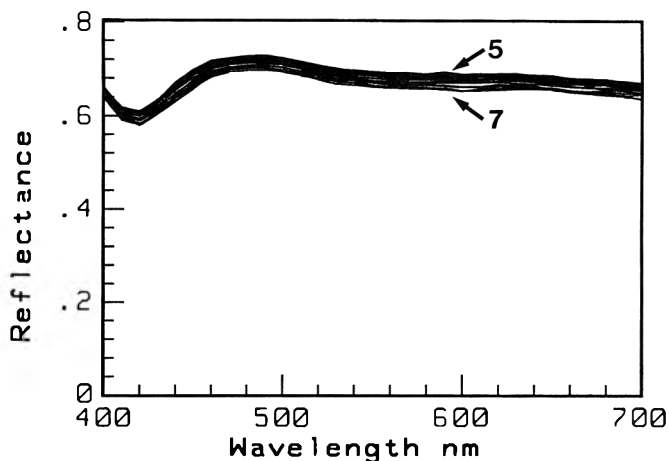
Fig. 8—A decrease in reflectance of naturally occurring severe PSE pork longissimus dorsi, when the pH was elevated in steps of pH 0.5 from pH 5 to 7 at 6 days postmortem.

Much work is needed to determine the extent to which each of the possible causes of pH-related pork paleness (precipitation, reflection and refraction) contributes to the final product, where color is important and complex (Brewer and Harbers, 1991). However, from my results, protein precipitation (Bendall, 1962; Bendall and Wismer-Pedersen, 1962; Fischer et al., 1979) appeared to be dominant in severe PSE. The magnitude of the myofibrillar effect (reflectance plus refraction) from pH 5 to pH 7 seldom exceeded reflectance 0.1 at any wavelength (Fig. 3, 6, 7), but could account for about 40% of the overall reflectance of normal pork taken to a low pH. However, severe PSE pork had a much higher reflectance than normal pork (compare Fig. 8 with Fig. 6). In the severe PSE pork (Fig. 8), the reversible effect of pH on myofibrils amounted to a reflectance of ≈ 0.04. Thus the balance of the reflectance implicates protein precipitation as the dominant cause.

CONCLUSION

A DIRECT EFFECT of pH was found on light scattering from myofibrils in pork. The effect was at least partly reversible and made a notable contribution to the overall reflectance of normal pork. It could be detected in severe PSE pork but was probably not the major cause of paleness.

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Antioxidative Maillard Reaction Products from Reducing Sugars and Free Amino Acids in Cooked Ground Pork Patties

A. J. BEDINGHAUS and H. W. OCKERMAN

──────── ABSTRACT ────────

Fifteen preformed Maillard reaction products (MRP) were prepared by refluxing 0.2M of three individual reducing sugars (glucose, xylose, and dihydroxyacetone) with 0.2M of five free amino acids (arginine, histidine, leucine, lysine, and tryptophan). MRP were added (3% v/w basis) to fresh ground pork patties prior to cooking and cooked to internal temperature 68°C and stored at 4°C for 10 days. Samples were analyzed for TBA value on day 0, 5, and 10. MRP were effective inhibitors of lipid oxidation in ground pork patties. The most effective were xylose-lysine, xylose-tryptophan, dihydroxyacetone-histidine, and dihydroxy-acetone-tryptophan when compared to controls. A significant interaction (P < 0.001) existed between reducing sugars and amino acids.

Key Words: antioxidant, pork, TBA, Maillard

INTRODUCTION

THE MAILLARD REACTION is of great importance to food science. The reaction is responsible for the "brown" color development in many cooked and baked foods. It is an extensive source of natural flavors and aromas as well as a principal resource for many synthetically derived aromas, flavors and colorants. The Maillard reaction has been studied by many researchers since the early 1900. Researchers have examined antioxidant activity of formed Maillard reaction compounds (Namiki, 1988) trying to identify "natural" compounds as food antioxidants for lipid applications in place of synthetic antioxidants (Kawashima et al., 1977; Dugan, 1980). The Maillard reaction is a result of interactions between amino-bearing groups, usually proteins or amino acids, and reducing sugars located (Reynold, 1965). These products are typically referred to as "Maillard reaction products" (MRP). Lipid foods have been relatively stable when the Maillard reaction was involved.

Hodge and Rist (1953) first reported lipid antioxidation effects of MRP for preserving vegetable oils. Other researchers have reported antioxidant properties of MRP (Patton, 1955; Griffith and Johnson, 1957; Zipser and Watts, 1961; El-Zeany et al., 1973; Itoh et al., 1975; Kato, 1973; Maleki, 1973; Kirigaya et al., 1968; Lingnert and Ericksson, 1981). The research work on MRP has primarily been conducted on model systems; only a few studies have been carried out in food systems (Lingnert et al., 1983).

The antioxidant effect of most formed MRP is related to the reducing sugar and amino compounds used as reactants (Lingnert and Eriksson, 1980a). Meat research studies that **directly** involve MRP as antioxidants are limited. Bailey et al. (1987) reported that the MRP mixture of glucose and histidine was effective at inhibiting lipid oxidation in cooked ground beef patties. Lingnert and Lundgren (1980) found rancidity development in frozen pork sausage was retarded with a MRP from glucose and histidine. The possible combinations of reducing sugars and amino acids that can be made into MRP are many but combinations studied in meat research studies are restricted. The browning products of MRP are generally considered safe for human consumption since they are made from natural products (Itoh et al., 1975).

Our objective was to evaluate the antioxidant effectiveness of several preformed Maillard reaction products (MRP) in cooked pork patties and to determine any effect the type of reducing sugar and amino acid had on lipid oxidation as measured by TBA values.

MATERIALS & METHODS

THE THREE SIMPLE MONOSACCHARIDE reducing sugars included: glucose, xylose, and dihydroxyacetone (DHA). They were selected based on chemical structure (one 3-carbon, one 5-carbon, and one 6-carbon sugar) and their antioxidant effectiveness as evidenced by previous lipid oxidation studies (Lingnert and Ericksson, 1981; Itoh et al., 1975; Tomita, 1971a, 1971b; Yamaguchi et al., 1964, 1981). The same reasons applied for the amino acids chosen; chemical structure diversity and their antioxidant effectiveness in prior lipid oxidation experiments (Lingnert and Ericksson, 1981; Itoh et al., 1975).

The amino acids were arginine, histidine and lysine (basic amino acids), leucine an aliphatic amino acid and tryptophan, an aromatic amino acid. Each was reacted with each individual reducing sugar to produce Maillard reaction products (MRP). A total of 15 MRP plus a control group (no MRP added) were studied. The MRPs were added to fresh ground pork muscle tissue prior to cooking.

The chemical reagents were: dextrose, monohydrate powder, (D-glucose), J.T. Baker Inc. (Phillipsburg, NJ); D-xylose, Fischer Scientific Co. (Fairlawn, NJ); dihydroxyacetone, L-arginine, L-histidine, L-leucine, L-lysine, L-tryptophan, 2-thiobarbituric acid, 1,1,3,3-tetramethoxypropane (TMP), Sigma Chemical Co. (St. Louis, MO); trichloroacetic acid (ACS, crystals) Jenneile Enterprises (Cincinnati, OH); meta phosphoric acid (AR, crystals) Mallinckrodt Inc. (Paris, KY).

Synthesis of Maillard reaction products (MRP)

The MRPs were prepared by refluxing 0.2M of each reducing sugar with 0.2M of each free amino acid in 100 mL double-distilled water. The MRP mixture was refluxed at 100°C for 20 hr in a 250 mL Pyrex™ flask coupled to a reflux condenser unit. Two replications were made for each synthesized MRP. The MRP mixtures, after refluxing, were stored in a cooler (2–4°C) until needed.

Ground pork muscle preparation

Whole fresh, vacuum-packaged pork loins (26–30 kg range) were purchased from local grocery stores. The pork loins were purchased 6–12 hr before they were utilized in the study and were ≈5–10 days postmortem. The pork loins were deboned, fat and lean tissue separated, and the meat block standardized to 18–20% fat content. The fat and lean were ground together through a 12.7 mm breaker plate (Stimpson grinder, model no. 5412, Stimpson Scale Co., Louisville, KY) and the coarse ground mixture was thoroughly mixed by hand. The meat-block was reground through a 4.8 mm sausage plate, and again manually mixed by hand. The fresh ground pork was prepared immediately prior to addition of the MRP and prior to cooking the pork patties.

Addition of MRP to Fresh Ground Pork

The fresh ground pork was divided into 180g lots for each MRP treatment and placed into Ziploc® 0.95 L polyethylene storage bags [177.8 mm × 203.2 mm, O_2 transmission rate = (420 cm³ O_2/mil thickness/645.2 cm²/24 hr/1 atm), 1.75 mils, Dow Brands Inc., Indianapolis, IN]. The MRP solution (3% v/w basis) was added to the 180g sample

Table 1—Cooking yield and TBA values of precooked ground pork patties held during refrigerated storage with incorporated MRP[a]

MRP	Cooking yield, %[b]	Thiobarbituric acid (TBA) value[c]			
		Day 0	Day 5	Day 10	SE[d]
Control	63.59[h,i]	0.361[A,f]	1.435[B,e]	1.600[B,e]	0.022
Glucose-arginine	61.0[i]	0.303[A,g]	1.177[B,f,g]	1.360[B,f,g]	0.043
-histidine	62.1[j,k]	0.296[A,g]	1.239[B,e,f]	1.466[B,e,f]	0.084
-leucine	63.3[h,i]	0.414[A,e]	1.307[B,e,f]	1.480[B,e,f]	0.029
-lysine	61.7[k,l]	0.268[A,h]	0.943[B,h]	1.124[B,h,i,j]	0.039
-tryptophan	62.8[i,j]	0.254[A,h]	0.951[B,h]	1.127[C,h,i,j]	0.017
Xylose-arginine	65.8[e]	0.274[A,h]	1.387[B,e]	1.510[B,e,f]	0.062
-histidine	65.7[e]	0.233[A,i,j]	0.918[B,h,i]	1.217[B,g,h]	0.070
-leucine	65.7[e]	0.205[A,k,l]	1.324[B,e,f]	1.486[B,e,f]	0.066
-lysine	66.3[e]	0.228[A,j,k]	0.809[B,i]	0.980[B,j,k]	0.167
-tryptophan	64.5[f]	0.166[A,m]	0.610[B,j]	0.849[C,k]	0.011
DHA-arginine	64.7[f]	0.344[A,f]	1.357[B,e,f]	1.607[C,e]	0.014
-histidine	64.2[f,g]	0.217[A,j,k,l]	0.620[B,j]	0.834[B,k]	0.086
-leucine	63.2[h,i]	0.168[A,m]	0.744[B,i,j]	1.004[B,i,j,k]	0.052
-lysine	64.0[f,g,h]	0.264[A,h,i]	0.957[B,h]	1.192[B,g,h,i]	0.064
-tryptophan	64.6[f]	0.203[A,l]	0.633[B,j]	0.816[B,k]	0.163
SE for values down the column	0.010	0.028	0.216	0.208	

[a] Duncans multiple range test was performed on these data.
[b] (Post-cook patty weight/precook patty weight) × 100.
[c] Total number of observations per treatment; n = 24.
[d] SE for TBA values across the row.
[e-m] Means with different lowercase superscripts within a column are significantly different (P<0.05).
[A,B,C] Means with different uppercase superscripts across the row for TBA values are significantly different (P<0.05).

and thoroughly mixed by hand. The 180g meat samples of each treatment were formed into two (90g) patties of uniform thickness and diameter (9.5 cm × 1.2 cm thickness) using a hand-held aluminum Burger Press™ (US Patent no. D191367, Heuck, Cincinnati, OH). The precooked weights were recorded after the patties were formed. The patties were cooked in an open-front Hotpoint broiler (Model no. 01B17, Hotpoint Inc., Chicago, IL) to an internal temperature of 68.3°C as determined by inserting a thermometer into the center of a representative sample of patties for each batch. A total of twelve pork patties were cooked per treatment batch. Immediately post-cooking, the patties were stored at room temperature (21°C) for 30 min before post-cook weights were recorded. The meat patties were sampled and analyzed on days 0, 5 and 10. The day 0 observation was analyzed immediately after the post-cook weight was recorded. The patties were stored in cardboard boxes in the meat cooler (2–4°C) in Ziploc® 0.95 L polyethylene storage bags and held in refrigerated (2–4°C) storage until TBA analysis of day 5 and 10 samples.

Thiobarbituric acid test method

The TBA test was carried out using a modified method of Witte et al. (1970). Samples (5g each) were obtained from each pork patty by cutting thin slices across the section width of the patty and placed in Cryovac® bags (12.7 cm × 30.5 cm, standard gauge, Cryovac Division, W.R. Grace Co., Simpsonville, SC) and homogenized in 50 mL solution of 20% trichloroacetic acid and 1.6% phosphoric acid. Each sample was stomached in a Stomacher 400 Lab Blender (Seward Medical, Ltd., London, England) for 2 min. Distilled water (50 mL, 4 ± 2°C) was added to the slurry and blended for 30 more sec in the stomacher. The mixture was filtered through Whatman® No. 1 filter paper. Distilled water was added to bring the filtrate to 100 mL volume. Filtered aliquot (5 mL) was mixed with 5 mL freshly prepared 0.02M 2-thiobarbituric acid solution in a glass test tube. The tubes were covered with parafilm and the solution mixed by inverting the tubes three times. The color was allowed to develop overnight in the dark for 15 hr at room temperature (21°C). The resulting color was measured for absorbance at 530 nm using a UV-VIS Hitachi Perkin-Elmer, Model 139, spectrophotometer (Hitachi, Ltd., Tokyo, Japan). The spectrophotometer was interfaced with an accessory detector unit, (a Hitachi Perkin-Elmer Photomultiplier unit, Hitachi, Ltd., Tokyo, Japan). Duplicate measurements were recorded for each pork patty sample analysis replication. Concentration of malondialdehyde was calculated from a standard curve using solutions of 1,1,3,3-tetramethoxypropane (TMP).

TBA value

The TBA value was calculated by multiplying the absorbance reading for the sample by the K value for extraction. The K value was calculated from standard curves and known dilutions as follows:

$$K \text{ (extraction)} = (S/A)(MW)(10^7/SW)(100/P)$$

The formula was obtained from Tarladgis et al. (1960) but some values used are different. The first term in the formula (S/A) represents the slope of the standard curve. The slope of the TBA value standard curve was 6.195×10^{-8}. The molecular weight (MW) of malondialdehyde (MA) is 72.03. The sample weight (SW) of sample analyzed was 5g. The average percent (P) recovery of enriched meat samples (5g) inoculated with 1500 µg, 1000 µg, and 500 µg of TMP was 71%. The calculated K value to be used in the TBA value calculation was 12.58. TBA values were calculated by multiplying absorbance by 12.58. TBA value was expressed as mg malondialdehyde (MA)/kg sample.

The moisture and crude fat of raw pork samples were determined by the oven drying method (100°C for 18 hr) and the Soxhlet ether extract method of Ockerman (1985).

The statistical analysis of TBA value data was determined with Statistical Analysis System (SAS Institute, Inc., 1988).

RESULTS & DISCUSSION

THE AVERAGE MOISTURE of raw ground pork in all TBA analyses was 61.6% ± 0.82 and fat was 19.1% ± 0.97. Calculated percentages for the moisture and fat data were based on six replications. Cooking yield and TBA values for all MRP treatments were compared (Table 1). Differences (P < 0.05) were found in cooking yield and TBA values among the various MRP treatments. A significant interaction (P < 0.001) existed between reducing sugars and amino acids. To simplify interpretation of data they were divided into groups. To discuss all 15 treatments inclusively as a group would make interpretation of results confusing because there were so many combinations. With the data considered in smaller groups, interpretations of results are easier to follow. The data of each MRP within each group was compared to data of the control group which served as the "standard."

TBA values of glucose/amino acid MRP treatment

Cooking yields were monitored because moisture content and water activity (a_w) are known to influence lipid oxidation (Chang and Watts, 1950; Fishwick and Zmarlicki, 1970). However, no relationship (P > 0.05) of cooking yield affected TBA values for the 15 MRP treatments (Table 1). The correlation coefficient of cooking yield vs all TBA values for day 0, 5, and 10 was r = 0.05 (plot not shown). Glucose-lysine and glucose-tryptophan were the two MRP that exhibited the least amount of lipid oxidation among the glucose-MRP and were the two most effective for the glucose-reducing sugar group. The control had TBA values 1.3–1.5 times greater than either the glucose-lysine or glu-

cose-tryptophan treatment for all three days of TBA analyses. The MRP reaction combinations of glucose with lysine and tryptophan have been shown to be effective in other lipid food systems (Lingnert and Ericksson, 1980a, b; Itoh et al., 1975; Tomita, 1971a, b; Yamaguchi et al., 1964). Kirigaya et al. (1969) reported MRP of glucose with histidine or arginine had strong antioxidant activity but results from our study did not indicate this for glucose-histidine. That MRP lowered TBA values throughout storage but they were not different (P > 0.05) from the control for Day 5 and 10. Lingnert and Lundgren (1980) showed MRP treatment of glucose with histidine lowered lipid oxidation in frozen pork sausage. Bailey et al. (1987) also showed glucose-histidine MRP treatment was an effective antioxidant in beef and pork.

TBA values of xylose/amino acid MRP treatments

The TBA values for the control group (Table 1) were greater than all xylose-amino acid combinations for each storage period. The xylose-tryptophan treatment had the lowest level of lipid oxidation among the xylose-MRP group but all three MRP treatments of xylose-histidine, xylose-lysine and xylose-tryptophan had lower (P < 0.05) TBA values when compared to the control. The control group TBA values were 1.9–2.0 times larger than TBA values from the xylose-tryptophan treatment for days 0, 5, and 10. Yamaguchi and Fujimaki (1974b) have shown xylose-arginine, xylose-histidine, and xylose-lysine effectively inhibited lipid oxidation in a linoleic acid system. Our results agree with Yamaguchi and Fujimaki's (1974a) finding except for the xylose-arginine treatment, which did not effectively inhibit lipid oxidation in the precooked pork patties. Lingnert and Eriksson (1980a) evaluated the antioxidative effect of MRP added to linoleic acid emulsion systems. They reported that xylose, reacted with arginine, histidine or lysine, effectively inhibited oxidation in this free fatty acid system. Lingnert and Eriksson (1980a) reported the xylose-arginine MRP had the strongest antioxidant activity of all MRP's tested. However, our results with xylose-arginine did not confirm that.

TBA values of dihydroxyacetone/amino acid MRP treatments

Almost every DHA-amino acid MRP treatment (Table 1) had lower TBA values than the controls (the only exception was day 10 for DHA-arginine with slightly higher TBA). The data for the DHA-MRP compounds spread over a wider range than the glucose or xylose-MRP groups. The DHA-histidine, DHA-leucine, DHA-lysine, and DHA-tryptophan MRP all had lower (P < 0.05) TBA values than the control for days 0, 5, and 10. The DHA-histidine and DHA-tryptophan MRP had the least lipid oxidation. The control TBA values for day 0, 5, and 10 were 1.6–2.3 times larger than the TBA value for DHA-leucine, DHA-histidine, and DHA-tryptophan MRP. Overall the DHA-MRP, with exception of DHA-arginine, were effective inhibitors of lipid oxidation in precooked pork samples. Kawashima et al. (1977) documented DHA and leucine MRP as an excellent MRP treatment for retarding lipid oxidation in safflower oil. Other DHA-MRP that Kawashima et al. (1977) tested were DHA reacted with lysine, leucine, isoleucine, valine, tryptophan, histidine, glycine, proline, alanine, methionine and asparagine. Itoh et al. (1975) reported DHA-leucine had superior antioxidant activity in preventing lipid oxidation in safflower oil and was more effective than the phenolic antioxidant butylated hydydroxyanisole (BHA).

TBA values of arginine and sugar MRP treatments

Control TBA values for day 0, 5, and 10 (Table 1) were ≈1.2 times larger than corresponding values for the glucose-arginine MRP. Glucose was the best reducing sugar in combination with arginine at reducing lipid oxidation in cooked pork patties.

TBA values of histidine and sugar MRP treatments

The DHA-histidine had the least lipid oxidation followed by the xylose-histidine MRP. The TBA values of control (Table 1) were 1.3–1.6 and 1.7–2.3 times larger than the xylose-histidine and DHA-histidine TBA numbers, respectively. Glucose, xylose, and DHA would be ranked as dihydroxyacetone > xylose > glucose in terms of decreasing antioxidant activity when reacted with histidine. Itoh et al. (1975) reported antioxidant activity of dihydroxyacetone as the most potent of various MRP tested, followed by xylose and glucose.

TBA values of leucine and sugar MRP treatments

The DHA-leucine MRP had the lowest amount of lipid oxidation among all leucine-MRP. The DHA-leucine MRP had TBA values 1.6–2.3 times smaller (Table 1) than control TBA values for all days. DHA was more effective than the two other reducing sugars when MRP were synthesized from leucine. Kawashima et al. (1977) reported DHA-leucine MRP had strong antioxidant activity in safflower oil.

TBA values of lysine and sugar MRP treatments

The xylose-lysine MRP had the least lipid oxidation in precooked ground pork among the lysine-MRP. The control group had TBA values for day 0, 5, and 10 that were 1.4–1.5, 1.6–1.8, and 1.3–1.5 times larger (Table 1) than TBA values for glucose-, xylose-, and DHA-lysine MRP, respectively. All three lysine-sugar MRP were effective at reducing lipid oxidation in cooked pork, however, xylose was the most effective. Lingnert and Eriksson (1980a) reported the MRP of xylose-lysine had potent antioxidant activity in linoleic acid emulsion systems.

TBA values of tryptophan and sugar MRP treatments

The control TBA values were 1.4–1.5 times larger than the glucose-tryptophan MRP (Table 1) for day 0, 5, and 10 TBA readings, and 1.8–2.4 times larger for both xylose- and DHA-tryptophan MRP. The xylose- and DHA-tryptophan MRP showed the least lipid oxidation among tryptophan-reducing sugar MRP for the duration of the experiment. Tomita (1971a, b) studied several MRP from glucose (none was made from xylose or dihydroxyacetone) and reported those from glucose and tryptophan were more effective than all others in antioxidant activity.

CONCLUSIONS

PREFORMED MAILLARD REACTION PRODUCTS (MRP) were effective at reducing lipid oxidation in precooked pork patties held during refrigerated storage. Most MRP had an antioxidant effect according to results of the TBA test. Some MRP had greater antioxidant effects than others. Most lipid oxidation occurred during the first five days of refrigerated storage with little or minimal oxidation after day 5. The four MRP that retarded lipid oxidation best were xylose-lysine, xylose-tryptophan, DHA-histidine, and DHA-tryptophan. The antioxidant potential of preformed MRP was related to reactant type. The reducing sugars with strong antioxidant potential were from the pentose and triose reducing sugars, xylose, and DHA. Amino acids with good antioxidant activity among the MRPs were histidine, lysine, and tryptophan. The antioxidant potential of MRP shows such materials can be made from natural precursors which can be used in precooked ground meats. Further research is needed to determine whether Maillard reaction products affect overall quality and sensory characteristics of precooked meat.

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Ms received 11/19/94; revised 4/6/95; accepted 5/12/95.

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Ms received 3/13/95; revised 4/20/95; accepted 5/23/95.

Acid/Base Status of Stress Susceptible Pigs Affects Cured Ham Quality

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─────────── ABSTRACT ───────────

Twenty-four halothane positive (HP) pigs received ammonium chloride (8 g/L), sodium bicarbonate (12.6 g/L) or water (HP control) ad libitum for 4 days and were then slaughtered. Hams from HP animals were pale, soft and exudative (PSE) and of inferior processing quality. Ammonium chloride induced a metabolic acidosis which further impaired cured ham quality (greater thaw purge, lower juiciness scores and less uniform slice appearance than comparable hams from control or bicarbonate treated HP hogs). Blood pH, carbonate and chloride concentration (day 3) were related to cured ham quality, indicating that acid-base balance may influence subsequent meat quality. Use of an alkaline salt provided little protection against PSE.

Key Words: PSE ham, acid-base, physiology, halothane+, processing quality

INTRODUCTION

PALE, SOFT EXUDATIVE (PSE) PORK is a problem in both domestic and export markets, with an economic cost to the Canadian pig industry in excess of $20 million annually. Incidence of PSE is influenced by many factors including genetics, extremes in temperature, and preslaughter handling. Manipulation of pre-slaughter diets has not been widely used to control PSE. Jones et al. (1988) and Murray et al. (1989) reported that fasting was effective in reducing PSE but could also reduce carcass yields. Potential for increased dark cutting with fasting has also been shown (Eikelenboom et al., 1991). Carbohydrate loading (sucrose) increased the incidence of PSE (Briskey et al., 1959).

Research by Ahn et al. (1992) and Boles et al. (1993) suggested that changing the acid/base balance of an animal prior to slaughter may potentially influence the severity of PSE. PSE is associated with an abnormally rapid drop in muscle pH immediately following slaughter. These researchers tested the hypothesis that feeding an alkaline salt in the antemortem period might provide a degree of protection against PSE and that an acid salt, such as ammonium chloride, might exacerbate the condition. Ahn et al. (1992) observed that dietary treatment with sodium bicarbonate delayed postmortem pH decline. However, they found no effect on fresh pork color and drip due to metabolic acidosis or alkalosis induced by feeding ammonium chloride or sodium bicarbonate. Boles et al. (1993) reported that fresh loin quality and sensory properties were negatively affected by acidosis induced by oral administration of ammonium chloride in halothane positive (HP) animals but sodium bicarbonate had no effect on fresh loin quality.

It has been well documented that PSE pork is of poor processing quality resulting in further processed meats with poor yields, poor bind and color (Wismer-Pedersen, 1960a, 1960b; Davis et al., 1975; Jeremiah, 1986; Honkavaara, 1988, 1990). Since up to 70% of pork is sold as further processed products (Industry, Science and Technology Canada, 1993), economic

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and quality losses in further processed items due to PSE are important. Initial investigations of acid/base status have considered fresh meat quality, in particular, quality of the loin. However, little is known regarding the effects of an oral acid or base load on processing characteristics of cured hams.

Our objective was to determine the effects of a preslaughter oral acid or base load on processing characteristics and eating quality of processed hams from halothane positive pigs.

MATERIALS & METHODS

Dietary and preslaughter treatment of animals

The experimental design was as previously reported (Boles et al., 1994). Briefly, 24 HP pigs were assigned to one of three dietary treatments: water (HP control), ammonium chloride (anhydrous) (8 g/L) or sodium bicarbonate (12.6 g/L). Animals were penned individually and water treatment was initiated 4 days prior to slaughter. These animals were from an HP herd with a predisposition to produce a high incidence of PSE (Murray et al., 1989). Blood pH and other parameters were assayed from blood collected from 18 animals on days 3 and 4 of treatment (Boles et al., 1994).

Slaughter procedures and sample collection

Animals were moved in groups of three to the meat laboratory at the Agriculture Canada Research Station in Lacombe, Alberta, where they were electrically stunned and then slaughtered following commercial practices. Along with the water-treated animals, eight halothane negative (HN) animals were slaughtered in a similar manner. Muscle pH of the longissimus, determined at 45 min, 24 hr and 48 hr postmortem, and other meat quality attributes were reported by Boles et al. (1993). At 24 hr postmortem, the ham was removed from the left side of each animal. Following removal of the shank at the femur/tibia joint, each ham was weighed, vacuum-packaged and frozen at −20°C. Hams were transported to the University of Saskatchewan and held in frozen storage (−20°C) for 3.5 mo prior to processing.

Ham processing

The 32 frozen hams (eight from each of four treatment groups) were further processed into bone-in, smoked, cured hams. Hams were thawed at 2°C for 96 hr. A sample of the exposed semimembranosus muscle (anterior end) was removed for pH determinations. After weighing, hams were pumped with brine to 115% green weight with two passes through a Pokomat pickle injector (Model P20/310. Max Hubner Ag, Berikon. Germany). The brine consisted of: 66.53% water, 16.67% sodium chloride, 13.33% dextrose (ADM Corn Processing, Clinton, IA), 3.00% sodium tripolyphosphate (HelaPhosP, Hermann Laue Spice Co., Scarborough, Ontario), 0.333% sodium erythorbate (Pfizer Chem Division, Kirkland, Quebec) and 0.133% sodium nitrite and was formulated to deliver 2.5% salt, 2% dextrose, 0.45% phosphate, 0.05% sodium erythorbate and 0.02% sodium nitrite for an expected cook yield of 100% green weight. Hams were weighed before and after injection to determine pump levels. Hams were netted with cotton stockinette, held for 3 hr at 2°C and then tumbled under vacuum for 5 min at 16 rpm to minimize injector needle marks. Each treatment group was tumbled separately, with additional brine added to the tumbler to achieve 115% green weight.

After holding overnight at 3°C, hams were reweighed, and then smoked and cooked in an Alkar smokehouse (Lodi, WI) according to a 14 hr cooking sequence. Temperature probes were inserted into the center of five hams. Once an internal temperature of 67°C was reached, the smokehouse was opened and the internal temperature of each ham was

Table 1—Effect of oral acid and base loading on processing properties of hams from stress susceptible pigs

| Attribute | Halothane negative control | Halothane positive | | | P > F |
		Ammonium chloride	Sodium bicarbonate	Control (water)	
pH (raw)	5.17	5.21	5.33	5.30	0.13
pH (cooked, cured)	6.08	6.06	6.12	6.16	0.26
Purge (thaw), %	1.1b	2.2a	1.6b	1.5b	0.04
Green weight, kg	7.2a	5.9b	6.1b	6.2b	0.001
Pump initial, % of green	114.4	114.3	114.3	114.7	0.82
Pump at 24 hr, % of green	112.6	111.2	111.3	111.5	0.08
Cook yield, % of green	99.6	96.1	96.4	97.2	0.054

a-b Means within the same row sharing a common letter are not significantly different at P < 0.05.

checked with a hand-held temperature probe. The internal temperature of most hams was 66–68°C (minimum of 65°C and maximum of 71°C). Hams were cooled for 8 hr, weighed to determine cooking shrink and vacuum-packaged in 3 mil nylon-poly barrier bags (oxygen permeability −53 cc/m²/24 hr). Hams were then held at 3°C until evaluation (within 2 wks).

The day before testing, a center cut ham slice (1.26 cm thick), parallel and posterior to the ischium, was removed from each of four hams (one from each treatment). These slices were vacuum-packaged and held overnight at 3°C in the dark. The remaining ham was subdivided into individual muscle groups (semimembranosus, SM; semitendinosus, ST; and biceps femoris, BF) and packaged.

Sensory evaluation

Sampling procedures were standardized for sensory and objective tests during preliminary work. During each taste panel session, samples from the same relative section of each ham were presented randomly to a ten member trained sensory panel. In addition to evaluations of eating quality, panelists scored visual properties of center cut ham steaks from each treatment.

Panelists were screened and selected following procedures of Cross et al. (1978) and the American Meat Science Association (AMSA, 1978). Initially, 18 panelists began the screening procedure. Training was given during 9 wks (17 sessions). The trained panel consisted of six females and four males and was heterogeneous with regard to age and socioeconomic status. Panel sessions were held 2 to 3 days a week in an atmospherically controlled sensory panel room equipped with 8 individual booths and red lights. Each panelist received two 1.3 cm cubes of ham from designated positions from each of four SM muscles (posterior half). Samples had been placed in small covered glass jars 30 min prior to each panel and were served at 21°C. Tap water was provided for rinsing between samples. Unsalted crackers also were provided.

The cubes of ham were scored for firmness (force required to compress the sample with the molar teeth), initial juiciness (based on two chews), overall juiciness (impression after continued chewing), and tenderness (based on time and effort required to masticate the sample) using 8-point descriptive scales (8 = extremely firm, juicy or tender; 1 = extremely soft, dry or tough). Saltiness and mealiness were scored on 6-point scales with 6 = not detectable and 1 = extremely salty or mealy). Ham flavor intensity and off-flavor intensity were scored on 8-point scales with 8 = extremely intense or not detectable and 1 = extremely bland or extremely strong.

The 10 member panel also individually viewed four vacuum-packaged center cut ham slices at each session. Assessments of appearance of the overall slice and of individual muscle groups (SM, ST, BF) were made under 50 footcandles of warm white fluorescent light. A diagram of a ham slice identifying the muscle groups was posted in the viewing booth. Color and color uniformity were scored on 6-point scales with 6 = very dark pink, and very uniform; 1 = very pale pink/not pink and very nonuniform.

Instrumental measures of color and texture

Prior to visual evaluations by the panel, color (L, a, b) of the vacuum-packaged ham slices was measured (in duplicate) using a Hunter colorimeter. Meat samples for the instrumental measure of tenderness (Warner Bratzler shear) were taken from the same relative position in each of the hams and cut adjacent to those used for sensory evaluation. Eight cores, 1.3 × 1.3 × 2.6 cm, were cut parallel to the muscle fibers from three muscle groups in each ham (SM-posterior half, BF-anterior and ST- posterior). Following equilibration to ambient temperature, cores were sheared once, perpendicular to the fiber direction, with a Warner

Bratzler shear attachment on a Food Technology Corp. Texture Test System (TP1, Reston, VA).

Chemical analyses

The pH of the semimembranosus muscle of the raw meat was measured prior to brine injection and of the cooked cured meat at the time of the sensory evaluation panels. Meat homogenates were prepared by blending 10g of meat with 90g of water. Homogenate pH was measured using a combination electrode and a Fisher Accumet 915 pH meter (Fisher Scientific, Ottawa, Ontario).

Experimental design and statistical analysis

Analyses of variance (ANOVA) were run using the General Linear Models procedure of SAS Institute, Inc. (1989). For yield data, color and shear values, a 1-way ANOVA was run (error mean square = 28 df). For sensory data, a split plot design was used with animal(treatment) (28 df) as the valid error term for testing the main effect of dietary treatment. Nonsignificant treatment by panelist interactions indicated that panelists were evaluating treatments in a similar manner. Least-squared means for each pig and attribute were calculated and merged with other data to determine simple correlation coefficients (Pearson's) among measurements.

RESULTS & DISCUSSION

Processing properties

The pH of hams, before or after curing and cooking was not affected by stress susceptibility or dietary treatment (Table 1). Boles et al. (1993) also reported that dietary treatment with ammonium chloride or sodium bicarbonate had no effect on ultimate pH of loin samples from the same hogs as used in the present study. However, they reported that the initial pH (45 min) in the loin of HP animals was ≈5.4 while halothane negative (HN) controls had an average inital pH of 6.0, indicating that the HP animals produced PSE carcasses while HN controls were of normal meat quality. The initial pH (45 min) was not determined for the hams in the present study. However, Warner et al. (1993) indicated that quality parameters determined on the loin were predictive of PSE conditions of ham muscles (SM, BF).

Water treatment and halothane sensitivity had slight effects on the processing properties of hams (Table 1). Hams from HP pigs treated with ammonium chloride had greater purge losses after thawing (P < 0.05) than comparable hams from HN controls, HP controls or HP hogs treated with sodium bicarbonate. Water treatment had no effect on brine retention or cook yields. Cured hams from the HP treatment groups weighed less (P < 0.05) and tended to have lower retention of brine and lower cook yields than HN samples.

Boles et al. (1993) reported that HP hogs treated with oral administration of ammonium chloride showed metabolic acidosis which affected sensory properties of loin roasts. However, water-holding capacity of ground loin muscle (Ahn et al., 1992), purge from fresh loin chops (Boles et al., 1994) or thaw losses of loin roasts (Boles et al., 1993) were not influenced by dietary ammonium chloride or sodium bicarbonate treatment.

Table 2—Effect of oral acid and base loading on sensory scores of hams from stress susceptible pigs

| Attribute[1,2] | Halo-thane nega-tive control | Halothane positive | | | |
		Ammo-nium chloride	Sodium bicarbo-nate	Control (water)	P > F
Slice color[c]	3.8	3.2	3.5	3.4	0.09
Slice uniformity[c]	3.5a	2.9c	3.2b	3.2b	0.04
SM color[c]	3.8	3.2	3.5	3.2	0.07
SM uniformity[c]	4.3	3.7	3.9	3.8	0.13
ST color[c]	3.8	3.6	3.7	3.9	0.78
ST uniformity[c]	3.1	2.4	2.9	3.0	0.08
BF color[1]	3.6	3.0	3.1	3.0	0.08
BF uniformity[c]	4.0	3.8	4.1	4.2	0.75
Firmness[d]	4.8	4.4	4.3	4.6	0.48
Initial juiciness[d]	5.2a	4.2c	4.6b	4.5b	0.05
Tenderness[d]	5.8	6.0	6.0	5.9	0.77
Overall juiciness[d]	5.4	4.5	4.9	4.9	0.08
Mealiness[c]	4.9	4.1	4.3	4.3	0.14
Saltiness[c]	2.9	3.4	3.3	3.4	0.36
Flavor intensity[d]	5.5	5.3	5.6	5.3	0.18
Off-flavor intensity[d]	7.5	7.4	7.6	7.5	0.61

[a-b] Means within the same row sharing a common letter are not significantly different at P < 0.05.

[c] Highest possible score = 6; 6 = very dark pink, very uniform, not detectable; 1 = very pale, very nonuniform, very mealy, very salty.

[d] Highest possible score = 8; 8 = extremely firm, juicy, tender, intense ham flavor, no off-flavor. 1 = extremely soft, dry, tough, weak, extremely strong off-flavor.

Several reports have indicated that PSE hams had higher drip/shrink losses during storage or distribution than uncured hams of normal quality (Kaufmann et al., 1978; Jeremiah and Wilson, 1987; Honkavaara, 1988). PSE meat is considered to have a more open texture which more easily absorbs pickle (Wismer-Pedersen, 1960a; Servini et al., 1986). However, Honkavaara (1988) observed that raw cured PSE hams retained less brine following curing and tumbling. Jeremiah and Wilson (1987) reported that PSE hams had lower brine retention, especially following frozen storage. Trout (1992), using a model cure system, observed that cure uptake was lower and cooking losses were higher for small (100g) PSE loin samples than meat from loins of normal quality.

It has been well established that cured PSE ham meat generally had lower smokehouse yields (Merkel, 1971; Kauffman et al., 1978; Jeremiah and Wilson, 1987; Honkavaara, 1988, 1990) which could result in economical losses. De Smet et al. (1992) reported that hams from HP animals had 2.3% lower technological yield than HN hams, likely due to development of PSE. In contrast, Boles et al. (1993) reported that dietary water treatment with ammonium chloride or sodium bicarbonate and halothane sensitivity had no effect on cooking losses of loin roasts from HP animals.

Sensory properties

Visual properties of ham samples were influenced both by halothane classification and dietary treatment (Table 2). In general, hams from HP hogs were less pink, and less uniform in color than hams from HN controls. Hunter color data (not presented) also indicated that cured hams from HP hogs tended to be less red (p = 0.052) in color than comparable HN control samples. Ammonium chloride samples received lower scores (P < 0.05) for color uniformity and tended (p = 0.09) to be less pink than ham slices from other HP water treatments, indicating that dietary ammonium chloride was detrimental to cured ham appearance. In contrast, Ahn et al. (1992) and Boles et al. (1993) found that dietary acid or base loading had no effect on color of raw meat from the loin.

In general, PSE hams had lighter cured color and were less uniform in appearance with more extensive two-toning than hams of normal quality (Wismer-Pedersen, 1960a, 1960b; Merkel, 1971). In contrast, De Smet et al. (1992) indicated that cured

hams from HN and HP phenotypes did not differ in color (Hunter values) and cured color stability.

Firmness, tenderness and flavor were not affected by halothane classification and dietary acid/base load (Tables 2). Shear values did not differ among treatments (data not presented). Such findings supported previous observations that PSE condition (Merkel, 1971; Davis et al., 1975) and halothane sensitivity (De Smet et al., 1992) had no effect on cured ham shear values. In contrast, Jeremiah (1986) reported that PSE and DFD ham samples were scored lower for flavor acceptability and tenderness than comparable samples from hogs of normal quality.

Initial juiciness scores were lowest for ham samples from ammonium chloride treated HP hogs and highest for HN controls (Table 2). Similar trends were observed for overall juiciness (p = 0.08) and mealiness (p = 0.14). Sensory properties of the hams were not affected by dietary bicarbonate. Honkavaara (1988) observed that PSE hams were crumbly and dry. Jeremiah (1986) reported lower juiciness scores for PSE hams.

Except for tenderness, the effect of dietary ammonium chloride on ham quality was similar (but less pronounced) to results previously noted for loin roasts (Boles et al., 1993). Those from HP animals treated with ammonium chloride were scored less firm, juicy, and more tender and mealy (P < 0.05) than comparable roasts from other HP or HN animals. Boles et al. (1993) also reported that dietary bicarbonate had no effects on palatability of loin roasts.

Relationships among sensory, quality and blood parameters

Sensory properties of cured hams were significantly correlated with ham instrumental measurements, fresh meat quality and blood (Table 3). Many correlations between sensory properties and ham instrumental measurements were statistically significant. Those hams, which were lighter in color, less red, with higher cooking losses had lower initial juiciness, overall juiciness, less cured pork flavor and greater mealiness and more off-flavors. These properties were indicative of PSE meat, however, quality defects were increased by the ammonium chloride treatment. As expected, shear values were negatively related to tenderness, but the correlation was relatively low (r = -0.44), likely due to the limited range of tenderness and shear values.

Eating quality (juiciness, mealiness, saltiness) of cured SM muscle was related to fresh loin quality parameters (pH$_{45}$, color and texture scores, Hunter color values), but in general, these relationships explained only 16-30% of the variation. Warner et al. (1993) concluded that quality parameters of the longissimus lumborum were predictive of fresh ham muscle quality but did not directly correlate loin and ham quality parameters. Lagaert et al. (1992) reported that sensory properties of cooked ham from HP and HN animals showed little relationship to differences in fresh meat quality.

The palatability of cured SM was significantly correlated with acid/base status of the animals before slaughter as indicated by blood data on day 3 of the trials. These relationships are of particular interest for several reasons. Correlations of data from only a subsample of 16 HP animals were possible. Further, while correlations of sensory data with blood data from day 3 of oral acid or base loading showed many significant correlations, correlation of blood data from day 4 (day of slaughter) showed little relationship to sensory properties (only three significant correlations, data not shown). It is possible that meat quality shows a time lag in response to blood parameters. Re-examination of relationships between loin quality parameters and blood data (Boles et al., 1994) from days 3 and 4 of oral treatment showed a similar response. Day 4 blood data significantly correlated with loin texture, Hunter a values and protein solubility (Boles et al., 1994), while blood data from day 3 showed significant relationships with several meat quality parameters (pH$_{45}$, drip loss, color scores, texture scores, Hunter a and b values).

Table 3—Pearson correlation coefficients (r) among palatability properties of cured ham slices, meat quality and blood measurements from stress susceptible pigs

Parameter	Firmness	Initial juiciness	Overall juiciness	Tenderness	Mealiness	Saltiness	Flavor intensity	Off-flavor intensity
Cured ham[a]								
Purge (thaw)	-0.12	-0.33	-0.28	0.22	-0.11	0.08	-0.18	-0.02
Cook yield	0.17	0.45**	0.48**	0.14	0.57***	-0.46**	-0.45**	0.44**
Hunter L	-0.26	-0.58***	-0.65***	-0.07	-0.68***	0.43*	-0.46**	-0.43**
Hunter a	-0.29	-0.57***	-0.53**	0.05	0.50**	-0.37*	0.38*	0.19
Hunter b	-0.30	-0.50**	-0.59***	-0.03	-0.64***	0.39*	-0.36*	-0.56***
shear	0.16	0.06	-0.09	-0.44*	-0.26	0.03	-0.08	-0.25
Fresh meat quality[b]								
pH45	0.20	0.41*	0.38*	-0.08	0.46**	-0.39*	0.19	0.15
Drip	-0.08	-0.35	-0.34	0.02	-0.39*	0.48**	-0.18	-0.26
Color	-0.09	0.50**	0.51**	0.16	0.44*	-0.54**	0.18	0.19
Hunter L*	-0.17	-0.43*	-0.41*	0.05	-0.34	0.33	-0.21	-0.05
Hunter a*	0.04	-0.29	-0.42*	-0.27	-0.51**	0.35	-0.27	-0.39*
Hunter b*	-0.11	-0.49*	-0.54**	-0.04	-0.50**	0.46**	-0.31	-0.28
Texture	0.09	0.49*	0.50**	0.01	0.49**	-0.48**	0.18	0.22
Blood[c]								
Veinous blood pH	-0.09	0.54*	0.45	0.06	0.28	-0.58*	0.46	0.00
Blood pCO2	0.09	0.37	0.43	0.06	0.60*	-0.29	0.58*	0.34
Blood HCO3	-0.01	0.57*	0.54*	0.06	0.50	-0.54*	0.64**	0.17
Blood base excess	-0.08	0.12	0.25	0.28	0.39	-0.23	0.24	0.22
Blood serum chloride	0.05	-0.65**	-0.64**	-0.05	-0.45	0.67**	-0.49	-0.37

[a] n = 32. Data for semimembranosus used. Standard Hunter Color values.

[b] n = 32. Measurements on the longissimus at time of slaughter (Boles et al., 1993). CIE Hunter color values.

[c] n = 16. Veinous blood parameters from day 3 of oral treatment. Blood pCO2 – veinous blood carbon dioxide content; HCO3 – calculated veinous blood bicarbonate; Base excess – calculated base excess of veinous blood (Boles et al., 1994).

*P < 0.05, **P < 0.01, ***P < 0.001.

Table 4—Pearson correlation coefficients (r) among visual properties of cured ham slices, meat quality and blood measurements from stress susceptible pigs

Parameter	Overall slice color	Slice uniformity	SM color	SM uniformity	ST color	ST uniformity	BF color	BF uniformity
Cured ham								
Purge (thaw)	-0.59***	-0.50**	-0.53**	-0.17	-0.43*	-0.22	-0.51**	-0.26
Cook yield	0.52**	0.46**	0.54**	0.46**	0.28	0.47**	0.42*	-0.16
Hunter L (SM)	-0.67***	-0.47**	-0.79***	-0.37*				
Hunter a (SM)	0.62***	0.41*	0.65***	0.09				
Hunter b (SM)	-0.70***	-0.58***	-0.72***	-0.43*				
Hunter L (ST)	-0.46**	-0.12			-0.78***	-0.33		
Hunter a (ST)	0.55**	0.34			0.51**	0.35		
Hunter b (ST)	-0.38*	-0.18			-0.55**	-0.46**		
Hunter L (BF)	-0.63***	-0.59***					-0.78***	-0.11
Hunter a (BF)	0.72***	0.53**					0.73***	0.16
Hunter b (BF)	-0.36	-0.29					-0.46**	0.14
Fresh meat quality[b]								
pH45	0.37*	0.38*	0.38*	0.47**	0.01	0.34	0.36*	-0.23
Drip	-0.22	-0.13	-0.26	-0.43*	-0.00	-0.10	-0.19	0.21
Color	0.40*	0.29	0.41*	0.48**	0.12	0.21	0.37*	-0.01
Hunter L*	-0.47**	-0.25	-0.50**	-0.25	-0.18	-0.15	-0.48**	0.10
Hunter a*	-0.26	-0.11	-0.32	-0.27	-0.22	-0.34	0.01	0.19
Hunter b*	-0.45**	-0.20	-0.51**	-0.24	-0.26	-0.31	-0.23	0.22
Texture	0.44*	0.39*	0.45**	0.44*	0.19	0.39*	0.35	0.01
Blood[c]								
Veinous blood pH	0.13	0.17	0.10	0.28	0.01	0.26	0.03	0.26
Blood pCO2	0.09	0.14	0.24	0.32	0.20	0.41	-0.17	-0.11
Blood HCO3	0.12	0.20	0.19	0.39	0.08	0.39	-0.08	0.11
Blood base excess	0.22	0.15	0.19	0.06	0.47	0.55*	0.09	0.42
Blood serum chloride	-0.10	-0.33	-0.14	-0.31	-0.12	-0.34	-0.10	0.07

[a] n = 32. SM = semimembranosus, ST = semitendinosus, BF = biceps femoris. Standard Hunter color values.

[b] n = 32. Measurements on the longissimus at time of slaughter (Boles et al., 1993). CIE Hunter color values.

[c] n = 16. Veinous blood parameters from day 3 of oral treatment. Blood pCO2 – veinous blood carbon dioxide content; HCO3 – calculated veinous blood bicarbonate; Base excess – calculated base excess of veinous blood (Boles et al., 1994).

*P < 0.05, **P < 0.01, ***P < 0.001.

Significant correlations between ham sensory properties and blood data clearly show that the acid/base balance of an animal influenced not only fresh meat quality, but also quality of further processed cuts such as cured hams. These relationships accounted for 25 to 45% of the variation in the data. At lower blood pH values, ham samples were less juicy and more salty. As blood carbonate levels decreased and chloride levels decreased (indicative of acidosis), juiciness scores were lower,

with less flavor and greater perceived saltiness. Any excess H+ in the blood is buffered by blood bicarbonate to form carbonic acid which ultimately becomes CO_2 and water (Valtin, 1983). The reciprocal relationship between chloride and bicarbonate has been previously reported (Valtin, 1983). Note that blood serum chloride positively correlated with perceived lack of saltiness, indicating as serum chloride levels increased, panelists perceived less saltiness in the meat.

Few researchers have reported the relationships between blood acid/base status and meat quality. Boles et al. (1993) observed that blood bicarbonate correlated with juiciness of loin roasts from HP animals treated with ammonium chloride and sodium bicarbonate. Tenderness and firmness from our results did not show any significant relationship with blood data. In contrast, Boles et al. (1993) found significant correlations between blood serum chloride and tenderness and firmness indicating that as chloride levels increased, cooked loin samples were more tender and less firm. Berends et al. (1993) evaluated 38 hematological and clinicochemical parameters from blood collected during exsanguination of 109 hogs and found no relationship between blood parameters (at the time of slaughter) and resulting meat quality.

In general, visual properties of cured hams correlated significantly with ham instrumental measurements and fresh meat quality but not with blood measurements (Table 3). Ham slice color and color uniformity were significantly related to purge (thaw) and cook yield, indicating that hams with less purge (thaw) and higher cook yields were more pink in color and more uniform in appearance. Hunter color values for the SM and BF were more closely correlated with overall slice visual properties than the ST, indicating the importance of these two muscles to perceptions of ham quality. As expected, panelist perceptions of cured ham color intensity correlated highly with Hunter color data.

Quality parameters determined on the loin (pH$_{45}$, color and texture scores, Hunter color values) (Boles et al., 1994) showed significant relationships to cured ham visual properties (Table 4). These relationships were generally low in magnitude (r^2 of 0.14–0.26), and indicated that properties of the loin did not fully predict the visual properties of cured hams from the same animals. In contrast to palatability properties, blood data (day 3) showed few significant relationships to visual color of ham slices from HP animals (Table 4).

CONCLUSIONS

ORAL LOADING OF AMMONIUM CHLORIDE caused acidosis and had negative effects on cured ham quality. Sodium bicarbonate had no effect on processing parameters or sensory properties of cured hams from HP animals. Correlations of ham quality parameters with blood data suggested that acid/base balance influenced meat quality. However, since all HP animals became PSE, any subtle effects of dietary treatment (especially of bicarbonate treatment) could have been obscured by the PSE condition. Furthermore, the very strong homeostatic mechanisms involved in maintaining acid-base homeostatis in the live animal would tend to counter the effects of oral loading with an alkaline salt. This issue requires further related research.

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Ms received 10/17/94; revised 3/24/95; accepted 4/8/95.

Survival of *Listeria monocytogenes* and *Salmonella typhimurium* and Quality Attributes of Cooked Pork Chops and Cured Ham after Irradiation

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——————— ABSTRACT ———————

Cooked pork chops (pumped with salt/polyphosphate brine or untreated) and cured hams were inoculated with *Listeria monocytogenes* and *Salmonella typhimurium*. The samples were irradiated at low (0.75 to 0.90 kGy) or medium doses (1.8 to 2.0 kGy), and each dose was delivered at either a low (2.5 M/min conveyor speed) or high (5.4 M/min) dose rate. Low-dose irradiation reduced *L. monocytogenes* by more than 2 log and *S. typhimurium* by 1 to 3 log. Pathogen populations and total plate counts (TPC) were reduced to undetectable levels by medium doses. No meat quality attributes were affected, and no dose rate effect was observed. Nitrite reduced ($P < 0.05$) both pathogens and TPC during 7°C storage in ham, especially when combined with low-dose irradiation.

Key Words: ham, pork chops, irradiation, sterilization, pathogens

INTRODUCTION

PRECOOKED MEAT has been used widely and is very popular among consumers for the convenience it provides. Precooked meat in a vacuum package should have an extended shelf life, because mild cooking decreases the initial microbial populations and vacuum packaging inhibits spoilage microorganisms and minimizes lipid oxidation (Jones et al., 1987; Stites et al., 1989).

Safety of such "microwave-ready" products has been considered. The potential *Clostridium botulinum* hazards associated with extended shelf life, refrigerated foods were reviewed by Conner et al. (1989). Sodium nitrite usually is used to lessen this risk in cured meat. However, addition of nitrite will affect meat color, so it is not suitable for cooked, uncured product. High doses have been shown to be necessary for *C. botulinum* control by irradiation (Annellis et al., 1977).

Precooked, vacuum-packaged, refrigerated meat products may also provide a potential growth environment for psychrotrophic organisms such as *Listeria monocytogenes* (Unda et al., 1991). This organism may survive and grow in atmospheres high in CO_2, such as in a modified atmosphere or vacuum packaging. Evidence for the presence of *L. monocytogenes* in cooked meat is less substantial because of the sensitivity of the organism to heat. In Australia, however, *L. monocytogenes* was detected on 93 of 175 samples (53%) of vacuum-packed processed meats from retail stores (Grau and Vanderlinde, 1992). Given the potential for growth of the organism at low temperature, the USDA-FSIS (USDA, 1989) has established a zero tolerance level for *L. monocytogenes* in cooked ready-to-eat meat products.

Salmonella spp. do not grow at > 47°C, and ideally, the organisms should not be found in cooked meat products. However, postcooking contamination may occur during chilling and before packaging, thus posing a hazard. *Salmonella* spp. grow optimally at 30–45°C and can grow slowly in the range of 5–15°C, but evidence has also shown that this organism can proliferate in fresh meats as low as 2.0°C and on shell eggs at 4.0°C within 6 and 10 days, respectively (D'Aoust, 1991). Thus, refrigeration

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temperatures do not guarantee the safety of contaminated cooked meat.

Ham, a cured product, can typically be stored longer than fresh meat because curing and smoking/cooking provide extended shelf life. Nitrite is used as a curing agent to give ham an attractive pink color and to suppress growth of *C. botulinum*. Salt and phosphate are also added to most cured and processed meat to provide flavor and texture. These compounds may inhibit growth of some microorganisms, depending on concentration in the product and on other factors such as pH and storage temperature (D'Aoust, 1991; McClure et al., 1991).

Irradiation provides a means to reduce use of chemical additives because it decreases the microbial load and eliminates some food pathogens. It also decreases the opportunity for postprocessing contamination if products are packaged before being irradiated. More research is needed for microwave-ready pork cuts and cured hams to determine any specific advantages of irradiation and to substantiate regulatory approval of this process.

There have been questions regarding possible effects of irradiation dose rate on destruction of microorganisms. It has been hypothesized that a high dose rate may not inhibit as many microorganisms as a low dose rate (Hayashi, 1991). Such difference may be due to recombination of radicals formed during irradiation at a high dose rate, rather than to reaction of the radicals with food components at a low dose rate. Also, high-dose-rate irradiation may result in an anoxic environment with fewer oxygen radicals. Some reports have shown no difference in effects on microorganisms between high dose rate and low dose rate, as demonstrated by comparing gamma irradiation (low dose rate) and electron-beam irradiation (high dose rate) (Hayashi, 1991). In our study, a single irradiation source (linear accelerator) and a fixed energy level were used to study dose rates. Different dose rates were achieved by changing power level and conveyor speeds. It is generally recognized that the energy level does not directly influence the antimicrobial effect of food irradiation. For example, Watanabe et al. (1988) reported that the energy (0.5-3.0 MeV) of electron beams did not influence the D_{10} value of *Bacillus pumilus* spores. Little work has been reported on potential differences in dose-rate effects on pathogens in meat products.

The objectives of our study were: to evaluate the effects of low or medium doses of irradiation on survival of *S. typhimurium* and *L. monocytogenes* in microwave-ready pork chops (pumped with salt/polyphosphate brine or unpumped) and cured boneless ham, to determine the effect of dose rate on survival of the organisms after irradiation processing, and to monitor product attribute changes such as pH, lipid oxidation, color, and odor in such products after irradiation and during storage.

MATERIALS & METHODS

Sample preparation

Chops. Fresh boneless pork loins were purchased from a commercial source and taken directly from the processing line to the Iowa State University Meat Laboratory. Thirty-six kg of brine solution, containing 3.6 kg of salt and 1.45 kg of polyphosphate, was prepared. Six loins ($\approx$ 32.6 kg) were weighed and pumped with brine to 110% of initial weight

by using a Townsend Model 1400 injector (Townsend Engineering Inc., Des Moines, IA) to achieve $\approx$1.0% salt and 0.4% polyphosphate concentration in the loins. The other six loins were used as unpumped controls. After pumping, all loins were cooked (without smoke) at 80°C, in an Alkar thermal processing oven (Alkar, Lodi, WI) to internal temperature 67°C. Cooking end point was determined by using a thermocouple inserted in the geometric center of the thickest loin. Loins were chilled at 2–4°C for 2 hr and sliced into 2.5-cm thick chops.

Hams. Hams needed for 3 replications of irradiation treatments were made as the same batch. Frozen boneless pork hams were thawed at 2–4°C, then weighed ($\approx$34 kg) and pumped with brine to 125% of initial weight by using a Townsend Model 1400 injector (Townsend Engineering Inc., Des Moines, IA). The curing solution (45 kg) was composed of water (36.3 kg), salt (5 kg), sugar (3 kg), polyphosphate (1 kg), sodium erythorbate (99.8g), and sodium nitrite (28.3g), with the concentration in the final product being 2.5%, 1.5%, 0.5%, 500 ppm, and 140 ppm, respectively. The hams were tumbled, stuffed, smoked, and cooked in an Alkar thermal processing oven (Alkar, Lodi, WI) with a preset cooking program. After achieving a core temperature of 68°C, the hams were chilled at 2–4°C for 72 hr. Hams were removed from casings and sliced into 5 mm-thick slices.

External inoculation and packaging

Cooked chops from pumped and unpumped loins and ham slices were individually divided into 3 subgroups for inoculation with *Listeria monocytogenes* or *Salmonella typhimurium*, as well as uninoculated controls. *Listeria monocytogenes* Scott A was grown in trypticase soy broth containing 0.6% yeast extract (TSB+YE) at 35°C for 24 hr until the stationary phase was reached. The culture was transferred to a fresh broth and incubated for 6 hr to the log phase (as determined by previous growth curve experiments). A 2-mL inoculum was transferred to a 10-mL TSB+YE broth and diluted in 1200-mL 0.1% peptone water, with a final concentration of $\approx$7 log cells/mL. By the same procedure, *Salmonella typhimurium* was grown in trypticase soy broth (TSB), and $\approx$ 6 log cells/mL was obtained.

For surface inoculation, chops or hams were dipped in the inoculum solution for 10 min and drained on sterilized racks for 2 min. Final cell concentrations were $\approx$ 6 log and 5 log cells of *L. monocytogenes* and *S. typhimurium*, respectively, per gram of meat. A high inoculum was used to make enumeration of surviving organisms easier. Samples were placed in high-barrier flexible pouches with O_2 permeability of < 2.5 cm³/645 cm²/24 hr at 23°C and 0% RH and water vapor transmission of < 1.0 g/645 cm²/24 hr at 38°C and 90% RH (Curlon™ 863 Saran, Curwood Inc. New London, WI). Vacuum packaging was completed by using a model A300 CVP machine (CVP System, Inc., Downers Grove, IL). After packaging, all samples were stacked in cardboard boxes and stored at 2–4°C for 12 hr before being irradiated.

Irradiation

Samples were irradiated at the Linear Accelerator Facility at ISU. The temperature of the products just prior to and immediately after irradiation was monitored by a thermocouple-datalogger (Li-can, Stanford, CT) and did not exceed 10°C. Chops and hams were placed in a single layer for irradiation but were irradiated separately because of differences in density and thickness of the products. Each inoculated or uninoculated group was further divided into five subgroups and assigned to different irradiation processing treatments. Low or high dose rates were produced by varying the power level (1 or 3 kW) and conveyor speed (M/min, for short or long exposure time) in the irradiator. For chops, samples were irradiated at 0.75 kGy or 2.0 kGy, each at low (3.3 or 1.3 M/min conveyor speed) or high (7.9 or 2.7 M/min) dose rates, with nonirradiated samples used as controls. Absorbed doses were measured by placing alanine pellets on both sides of a chop. An Electron Paramagnetic Resonance (EPR) instrument was used to determine the absorbed dose (Bruker Instruments, Inc., Billerica, MA). The instrument was calibrated with standard dosimeters provided by the National Institute of Standards and Technology (NIST).

Ham slices were irradiated at 0.90 kGy or 1.8 kGy, each at low (3.6 or 1.7 M/min) and high (8.2 or 3.6 M/min) dose rates. A cutting board was placed on the top of ham samples to achieve sample thickness similar to the chops. To avoid crushing the pellets which were used for the chops, absorbed doses were measured by placing GAF chromic films on both sides of a ham slice. The films were each enclosed in a high-barrier paper pouch to protect them from moisture. The film was nylon based, with a thin layer of radiochromic film intertwined. Radiation activated color change was measured by using a FWT 100 reader (Farwest Tech-

nologies, Inc., Goleta, CA), which was calibrated with data in the EPR instrument. Reported doses are averages of doses absorbed at the upper surface and the lower surface of the samples. Variation between minimum and maximum doses was ± 0.1 kGy for low doses and ± 0.2 kGy for medium doses, and was the same for both the chops and ham slices.

All combinations of treatments for chops and hams were analyzed immediately after irradiation (day 0). Samples were also stored at 7°C for 7 days to simulate consumer storage, followed by temperature abuse at 25°C for 2 days to simulate mishandling of products after purchase. Preliminary studies have shown that storage of processed meats at 2–4°C (simulated wholesale/retail storage) increased TPC by only $\approx$1.0 log CFU/g; therefore, 7°C was used to represent what might be the temperature of a household refrigerator. Storage conditions also served to accelerate the storage stability evaluation.

Physical and chemical analyses

Measurements of pH, lipid oxidation (TBA), and color were conducted on uninoculated samples except for products for which the total plate counts showed obvious spoilage ($>10^7$ CFU/g) or when obvious off-odor developed. Sample pH was measured by using a slurry of 10 g of meat with 100 mL distilled water, a pH meter (Fisher Accumet Model 925, Fisher Scientific, Pittsburgh, PA), and a sealed combination electrode with silver/silver chloride reference (Omega Engineering, Inc., Stamford, CT). Measurement of thiobarbituric acid (TBA) values, an index of lipid oxidation, was performed by the method of Tarladgis et al. (1960) for chops and modified as appropriate for cured hams. Duplicate readings were recorded for each sample for pH and TBA tests. Color (L, a, and b) measurements were made with a HunterLab Labscan spectrophotometer (Hunter Associated Laboratories Inc., Reston, VA). An average value was obtained from 3 random locations on each sample surface.

Microbiological analyses

Packages from each treatment were aseptically opened on sampling days, and a 25-g sample was removed with a sterile scalpel. The sample comprised the top 0.5 cm of surface tissue. Samples were homogenized separately for 2 min in a sterile bag containing 225 mL of sterile 0.1% peptone water in a Stomacher lab blender (Model 400; Tekmer™ Co., Cincinnati, OH). To determine the presence of each of the two pathogens, direct plating onto selective media was done. A Most Probable Number (MPN) method was also done to enumerate each pathogen in samples where the number of bacteria were too few to detect by direct plating. *Salmonella typhimurium* cells were enumerated by serially diluting the sample in 0.1% peptone buffer, plating onto Xylose Lysine Deoxycholate (XLD, DIFCO) plates and incubating for 48 hr at 35°C. Typical colonies were enumerated with a Quebec colony counter (American Optical Co., New York), identified by growth characteristics in triple sugar iron (TSI) slants, and their identity confirmed by an Enterotube II system (Roche Diagnostic Systems, Montclair, NJ). For samples where no salmonellae could be detected by direct plating, a three-tube MPN procedure with Selenite broth (DIFCO) was followed (Flowers et al., 1992). Positive tubes were streaked onto SLD plates, incubated for 48 hr at 35°C, and typical colonies were identified as above.

Listeria monocytogenes cells were enumerated by serially diluting the samples in 0.1% peptone buffer, plating onto Modified Oxford Agar plates and incubating for 48 hr at 35°C. Typical colonies were enumerated, identified by Gram stain, catalase, oxicase, tumbling motility at 25°C, and their identity confirmed by Micro-ID Listeria (Organon Teknika, Durham, SC). For samples where no listeriae were detected by direct plating, a 3-tube MPN procedure with University of Vermont Modified broth (UVM, Difco) was followed (McClain and Lee, 1989). Growth from positive tubes was inoculated onto Modified Oxford Agar plates, incubated for 48 hr at 35°C, and typical colonies were identified as above.

Serial dilutions were also prepared according to recommended microbiological procedures (Vanderzant and Splittstoesser, 1992), surface-plated onto Plate Count Agar (PCA, DIFCO), and incubated aerobically at 25°C for 48 hr to enumerate total plate counts. In this case, no MPN procedure was used when counts were ≤ 2 log CFU/g. Total plate counts were estimated as closely as possible when low counts occurred.

Sensory color and odor evaluation

Only uninoculated samples were used for color and odor evaluations, which were conducted at day 0. A 10-member sensory panel evaluated
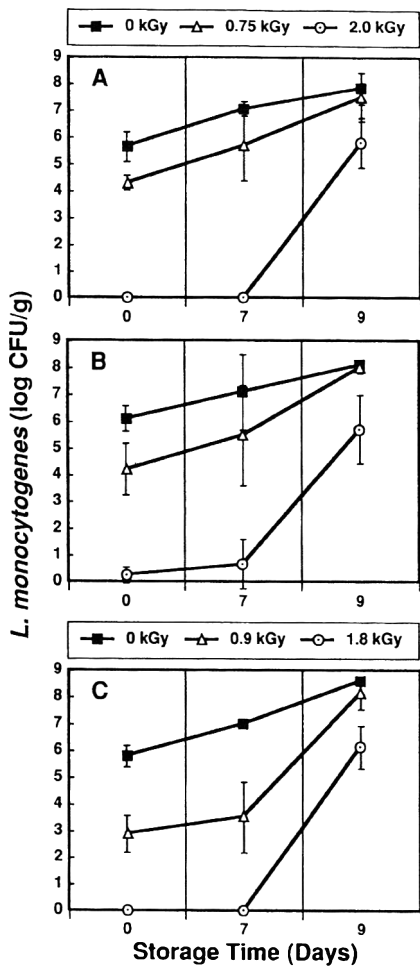
Fig. 1—Growth of *L. monocytogenes* in unpumped (A) or pumped (B) cooked chops, and hams (C) treated with irradiation, and stored at 7°C (day 0-7), then 25°C (day 7-9). Bars represent standard deviation of triplicates.
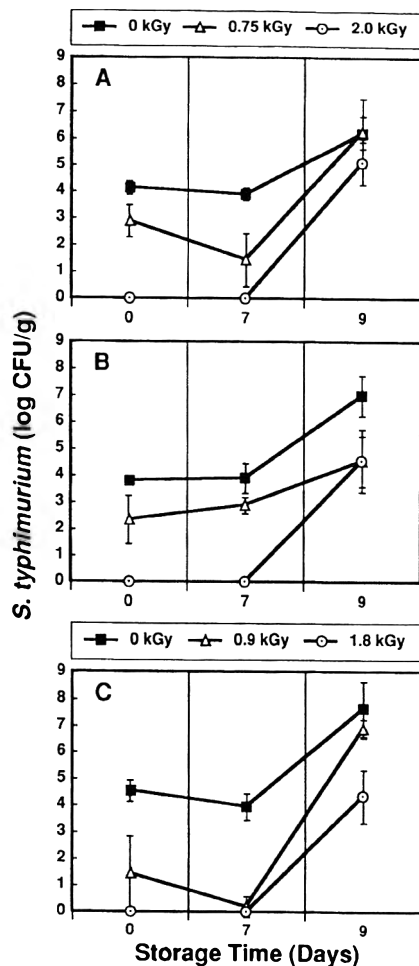
Fig. 2—Growth of *S. typhimurium* in unpumped (A) or pumped (B) cooked chops, and hams (C) treated with irradiation, and stored at 7°C (day 0-7), then 25°C (day 7-9). Bars represent standard deviation of triplicates.
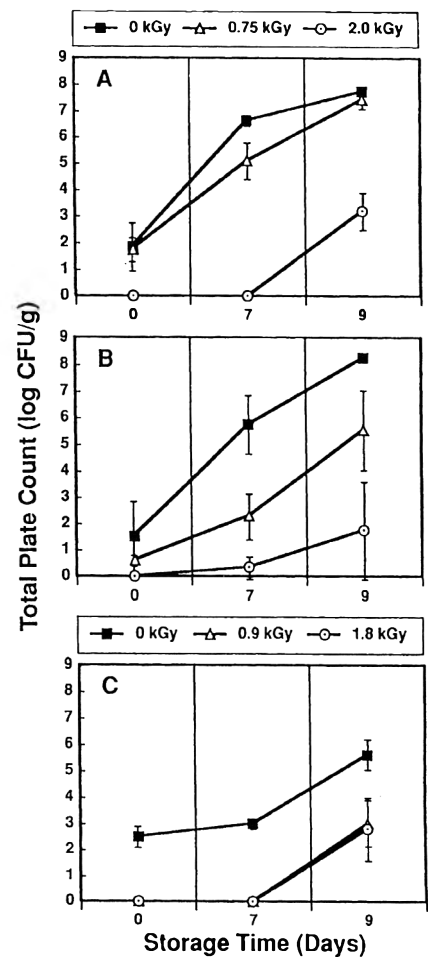
Fig. 3—Total plate counts in unpumped (A) or pumped (B) cooked chops, and hams (C) treated with irradiation, and stored at 7°C (day 0-7), then 25°C (day 7-9). Bars represent standard deviation of triplicates.

each sample by using a 5-point descriptive scale for surface color (1 = dark gray; 5 = red) and odor (1 = pleasant, no off-odor; 5 = extreme off-odor). Panelists were selected from faculty and graduate students in the Animal Science department who were experienced with sensory panels. No training of panelists was included. Chops or hams were tempered at room temperature (≈23°C) for 2 hr, unwrapped, and placed in Ziploc® (DowBrands L.P., Indianapolis, IN) pouches for immediate evaluation. Ten chops or five ham samples were evaluated at each session. Chops and hams were evaluated in separate sessions.

Statistical analyses

The experiment with cooked chops included 30 treatment combinations: two brine treatments, three inoculation treatments, and five irradiation treatments (2 × 3 × 5 = 30). Measurements of pH, TBA, Hunter color L, a, b and sensory evaluation were conducted only on uninoculated samples; thus including 10 (2 × 1 × 5) treatment combinations. Microbiological, physical, and chemical analyses were conducted at day 0, 7, and 9, and sensory evaluation was conducted at day 0. Three replications were conducted. The total number of chops used was 390 [(30 × 3 + 10 × 3 + 10 × 1) × 3].

The experiment with hams included 15 treatment combinations: three inoculation treatments, and five irradiation treatments (3 × 5 = 15). The same sampling design was used as for chops; therefore, the total number of ham slices used was 195.

Microbiological data were transformed into logarithms of the number of colony-forming units/g ($\log_{10}$ CFU/g). Average data and standard errors were calculated from three replications. Different sampling times (day 0, 7, and 9) were analyzed separately because different temperatures were used for storage. Physical, chemical, microbiological, and sensory

data were analyzed by using the Statistical Analysis System (SAS Institute Inc., 1986). The analysis of variance (ANOVA) procedure was used to detect significance of replications, brine pumping (chops only), dose, and brine by dose (chops only); dose rate and other combination effects were also evaluated. If no significant difference existed between low and high dose rates, data were combined (averaged) for analyses. Comparisons of means were based on Duncan's multiple range test.

RESULTS & DISCUSSION

Dose rate effects on microorganisms and quality attributes

No dose-rate effect was observed for the range of rates used (data not shown). Technically, using a single radiation source means that the dose rate differences that can be obtained are relatively limited. There was more than a twofold difference between the low and high dose rates used, but the dose rates did not result in a difference in numbers of survivors. Further investigation on the effect of dose rate on survival of microorganisms might be informative if rates differed by a wider margin than those we used.

Because no significant difference resulted from the two dose rates, the data were combined and analyzed for other treatment effects on each pathogen.

Effects of irradiation on microorganisms

Listeria monocytogenes. Irradiation was effective in reducing *L. monocytogenes* on inoculated chops or hams. For chops, ir-

radiation at 0.75 kGy reduced (P < 0.05) populations by 2 log CFU/g; but after storage for 7 days, the number of *L. monocytogenes* colonies was not different (P > 0.05) from unirradiated controls. Temperature abuse allowed *L. monocytogenes* to proliferate to 8 log CFU/g (Fig. 1–A, B). Low dose (0.9 kGy) irradiation was more effective for reducing *L. monocytogenes* on hams than chops (Fig. 1–C). *L. monocytogenes* was reduced by 3 log CFU/g on hams (Fig. 1–C), as compared with a 2 log CFU/g reduction on chops (Fig. 1–A, B). This effect was maintained after 7 days storage, with the number of cells not exceeding 4 log CFU/g (Fig. 1–C). However, the organism grew well after temperature abuse, at 25°C, reaching the same number on both hams and chops of ≈ 8 log CFU/g.

Medium-dose irradiation resulted in an even greater reduction in number of survivors of *L. monocytogenes*. The organism on either chops or hams was reduced (Fig. 1) to virtually undetectable levels after irradiation with 2.0 kGy. However, this dose did not eliminate *L. monocytogenes* because some cells were able to recover when the temperature was elevated to 25°C. Even so, the final population on both products after 9 days was less than those on controls or on samples exposed to low-dose irradiation.

No brine effect was observed for chops immediately after irradiation, or after extended storage (Fig. 1–A, B). Concentrations of salt and polyphosphate in pumped chops were 1.0 and 0.4%, respectively. This salt concentration was not high enough to affect growth of *L. monocytogenes*. This was expected because this organism can survive in trypticase soy broth containing 25.5% NaCl for more than 18 wk (Shahamat et al., 1980). Junttila et al. (1989) determined that salt content of Finnish fermented sausages at 3–3.5% had little effect on *L. monocytogenes*. From a study on processed meat products, Glass and Doyle (1989) suggested that *L. monocytogenes* generally grew well on meats near or above pH 6 and poorly or not at all on products near or below pH 5. The pH of the chops (Table 1) was 5.7–5.9 at day 0. The low levels of salt and polyphosphate probably explain why no significant difference (P > 0.05) was detected in growth of *L. monocytogenes* between unpumped and pumped samples. Temperature, pH, and sodium chloride can be important in the growth of *L. monocytogenes* at appropriate concentrations (McClure et al., 1991).

The number of *L. monocytogenes* increased by only 1 log after incubation at 7°C, on both nonirradiated and irradiated (0.9 kGy) hams. This suggested that this temperature contributed to inhibition of growth. Lower temperature usually is beneficial to this organism because it can survive in ground beef at 4°C for 2 wk (Johnson et al., 1988) and at temperature as low as 1.1°C (Junttila et al., 1988). In a review by Radomyski et al. (1993), results from an experiment with artificially inoculated samples showed the D_{10} value of *Listeria spp.* was between 0.4–0.6 kGy. From this information, the medium doses (2.0 or 1.8 kGy) we used should have reduced counts by 5 log CFU/g at most, but reduction by almost 6 log CFU/g was observed. Vacuum packaging and refrigeration may enhance the inhibitory effect of irradiation (Patterson, 1988), although other studies have reported no difference in survivors after irradiation of meat packaged in air vs. vacuum (Varabioff et al., 1992). When comparing hams with pumped chops, a higher salt concentration (2.5% vs 1.0%) and addition of nitrite to ham probably was responsible for the greater inhibition of *L. monocytogenes*, especially when low-dose irradiation was applied. Buchanan et al. (1989) concluded that nitrite could have significant bacteriostatic effects on *L. monocytogenes*, particularly if used in conjunction with low pH, vacuum packaging, high NaCl concentrations, and adequate refrigeration.

Salmonella typhimurium. Irradiation was also effective in reducing S. typhimurium on inoculated chops and hams. Low-dose (0.75 or 0.90 kGy) irradiation reduced the numbers by 1 log for chops and by 3 log for hams (Fig. 2). Unlike *L. monocytogenes*, the number of *S. typhimurium* survivors did not increase after 7 days storage at 7°C. *S. typhimurium* was

Table 1—Selected characteristics of irradiated unpumped (U) and pumped (P) cooked chops during storage at 7°C (day 0–7) and 25°C (day 7–9)

| | Days of storage | Irradiation dose (kGy) | | | | | |
| | | 0.0 | | 0.75 | | 2.0 | |
		U	P	U	P	U	P
pH	0	5.71[a]	5.81[a]	5.93[a]	5.80[a]	5.86[a]	5.81[a]
	7	5.92[a]	5.86[a]	5.84[a]	5.85[a]	5.88[a]	5.85[a]
	9	5.49[a]	5.69[bc]	5.57[c]	5.88[ab]	5.98[a]	5.91[ab]
TBA	0	0.57[ab]	0.33[b]	0.77[a]	0.34[b]	0.74[ab]	0.42[ab]
	7	1.81[a]	0.43[b]	1.68[a]	0.39[b]	1.27[ab]	0.39[b]
	9	0.42[a]	0.38[a]	0.48[a]	0.34[a]	0.45[a]	0.35[a]
L value	0	66.2[a]	65.1[a]	65.0[a]	63.5[a]	63.9[a]	66.1[a]
	7	65.2[a]	65.1[a]	63.2[a]	65.3[a]	65.8[a]	62.4[a]
	9	66.7[a]	64.2[a]	63.9[a]	65.5[a]	63.6[a]	63.6[a]
a value	0	2.7[c]	2.9[c]	3.4[bc]	3.6[ab]	4.3[a]	4.3[a]
	7	3.2[b]	3.0[b]	3.3[b]	3.2[b]	4.2[a]	4.3[a]
	9	4.0[ab]	3.5[ab]	4.1[a]	3.2[b]	3.6[ab]	3.7[ab]
b value	0	11.5[a]	9.8[bc]	11.0[a]	9.7[c]	10.4[b]	9.2[d]
	7	11.7[a]	10.4[b]	11.4[a]	10.1[bc]	10.9[ab]	9.6[c]
	9	11.1[a]	9.8[b]	10.9[a]	10.2[b]	11.1[a]	10.1[b]

[a-d] Values within each row with the same superscripts are not significantly different (p > 0.05).

effectively inhibited by refrigeration temperature because this organism is a mesophile, preferring room temperature over refrigeration. *S. typhimurium* has a comparable D_{10} value (0.4–0.7 kGy) to *L. monocytogenes* (0.4–0.6 kGy) (Radomyski et al., 1993); thus, medium doses such as those used in our study (2.0 or 1.8 kGy) reduced populations to undetectable levels on both products. Combined with refrigeration at 7°C, no *S. typhimurium* was detected even after 7 days storage. Because these medium doses did not kill all *S. typhimurium*, survivors still proliferated when the temperature was elevated to 25°C.

There was no significant (P > 0.05) effect on *S. typhimurium* due to brine injection in the chops. Higher salt concentration and addition of nitrite in hams enhanced inhibition of *S. typhimurium* during storage, especially when low-dose irradiation was used. After storage at 7°C for 7 days, low-dose irradiation decreased *S. typhimurium* on hams close to undetectable levels (Fig. 2–C), whereas 1-3 log CFU/g of the organism was found on chops after the same storage time (Fig. 2–A, B).

Uninoculated samples. No *L. monocytogenes* or *S. typhimurium* survivors were detected on uninoculated chops or hams. Precooking "microwave-ready" chops provided for good microbiological quality of these products, which was evidenced by low (2 log CFU/g or less) initial TPC. Such low numbers were estimated as closely as possible, however, no additional efforts were made to determine the low TPC numbers more precisely. Low-dose (0.75 kGy) irradiation resulted in no change (P > 0.05) in TPC compared with controls, but a reduction (P < 0.05) of TPC was observed during storage at 7°C (Fig. 3–A, B). No reduction was observed for unpumped samples after temperature abuse, while a 2-log reduction was observed for pumped samples. This inhibition may have been due to brine injection. Thus, injection of salt and phosphate at low levels, used primarily for moisture retention and flavor, seemed to provide some assistance for extending shelf life, when combined with low-dose irradiation and refrigeration. Medium-dose irradiation at 2.0 kGy decreased TPC to undetectable levels, which was lower (P < 0.05) than for controls (nonirradiated). Irradiation at this level showed an effect during extended storage and especially after temperature abuse, with samples irradiated at 2.0 kGy showing a decrease in total populations of 5 and 6 log CFU/g on unpumped and pumped chops, respectively, compared with controls.

Ham is cured with salt, phosphate, and nitrite and is also cooked; thus, a low initial TPC (2.5 log CFU/g) was expected (Fig. 3–C). The combined treatment of vacuum packaging and refrigeration temperature (7°C) can further inhibit cell growth. When low (0.9 kGy) or medium (1.8 kGy) doses of irradiation were applied, all organisms were reduced to undetectable levels even after storage at 7°C for 7 days. However, when storage temperature was 25°C, some cells were still able to grow.

Table 2—Selected characteristics of irradiated hams during storage at 7°C (day 0–7) and 25°C (day 7–9)

| | Days of storage | Irradiation dose (kGy) | | |
		0.0	0.90	1.8
pH	0	6.24[a]	6.26[a]	6.25[a]
	7	6.20[a]	6.23[a]	6.22[a]
	9	6.26[a]	6.34[a]	6.30[a]
TBA	0	0.15[b]	0.15[b]	0.17[a]
	7	0.48[a]	0.43[a]	0.43[a]
	9	0.39[a]	0.43[a]	0.35[a]
L value	0	56.2[a]	52.6[b]	53.9[ab]
	7	57.0[a]	54.9[a]	56.5[a]
	9	56.7[a]	55.4[a]	56.1[a]
a value	0	8.0[a]	7.9[a]	7.5[a]
	7	7.8[a]	8.1[a]	7.9[a]
	9	8.4[a]	8.7[a]	8.5[a]
b value	0	5.0[a]	4.0[a]	4.4[a]
	7	4.4[a]	4.1[a]	4.4[a]
	9	4.8[a]	4.8[a]	4.9[a]

[a,b] Values within each row with the same superscripts are not significantly different (p > 0.05).

Table 3—Sensory color and odor characteristics of irradiated unpumped (U) and pumped (P) cooked chops

| | Irradiation dose (kGy) | | | | | |
| | 0.0 | | 0.75 | | 2.0 | |
	U	P	U	P	U	P
Color	2.4[a]	2.1[b]	2.6[a]	2.3[ab]	2.6[a]	2.6[a]
Odor	1.6[d]	1.7[cd]	2.1[bc]	2.6[a]	2.6[a]	2.3[ab]

[a-d] Values within each row with the same superscripts are not significantly different (p > 0.05).

Table 4—Sensory color and odor scores for irradiated hams

| | Irradiation dose (kGy) | | |
	0.0	0.90	1.8
Color	4.0[a]	3.6[a]	3.9[a]
Odor	3.4[a]	3.2[a]	3.5[a]

[a] Values within each row with the same superscripts are not significantly different (p > 0.05).

Most microbial flora on vacuum packaged meat products and/or irradiated samples are lactic acid bacteria (Lebepe et al., 1990). Although these bacteria are not pathogens, a high TPC is still considered an index of quality deterioration. Therefore, refrigeration is required to achieve a long shelf life of these products even when irradiation is applied at low to medium doses.

Irradiation effects on quality attributes

Irradiation did not affect pH, TBA, instrumental color or sensory color, or odor attributes for chops or hams. Most pH values of chops and hams were about 5.7–5.9 (Table 1) and 6.2–6.3 (Table 2) respectively, indicating that, except for phosphate effects, pH was not affected by factors such as brine injection, curing agents, irradiation, or storage conditions.

TBA value is an index of lipid oxidation, and indicates rancidity for meat and meat products. Pumped chops had (P > 0.05) TBA values similar to unpumped chops at day 0 and 7 (Table 1). TBA values of most samples were within acceptable ranges (< 1.00), indicating product stability in terms of lipid oxidation. Unda et al. (1991) showed 5 wk of no change in lipid oxidation for microwave-ready beef roasts. Most TBA values at day 9 were lower than at day 7, suggesting that microbial degradation of malonaldehyde and other thiobarbituric acid reactive substances (TBARS) occurring in meat samples may have caused lower TBA values (Moerck and Ball, 1974). Nitrite can also retard lipid oxidation (Pearson and Tauber, 1984). This was observed in all TBA values for hams at day 0, with values below

0.17 (Table 2). These values were lower than those for pumped chops (0.32–0.47) (Table 1). TBA values for hams remained below 0.5 throughout storage. No dose or dose rate effect (P 0.05) was seen in TBA values, indicating that irradiation as high as 1.8 kGy did not affect this chemical reaction.

Color evaluation, conducted by HunterLab and sensory panel, indicated no consistent difference (P > 0.05) between samples. Color evaluation by panelists did not detect differences between treatments except that pumped chops had the lowest value at day 0 (Table 3). No change occurred in color 'L' or 'b' values. Surface color of hams was not changed by any level of irradiation. Hunter L, a, and b values (Table 2) and the color score from sensory panels (Table 4) showed no difference (P > 0.05) between any samples.

Odor evaluation of chops showed that some irradiated samples emitted a detectable off-odor compared with controls, but this effect was not dose dependent (Table 3). The predominant lactic acid bacteria within such a vacuum packaging environment may emit some "sour" odors. No odor difference (P > 0.05) in hams (Table 4) was detected by panelists.

CONCLUSIONS

IRRADIATION was effective for reducing L. monocytogenes, S. typhimurium, and total plate counts on "microwave-ready" chops and cured hams, especially at medium doses (1.8–2.0 kGy). Accompanied by refrigeration, irradiation should ensure the safety of these products and should extend their shelf life. Dose rate had no effect on the organisms. Irradiation at appropriate levels was also more effective in combination with the salt and nitrite added for bacterial control in cured ham.

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Supercritical Carbon Dioxide Extraction of Androstenone and Skatole from Pork Fat

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——————— ABSTRACT ———————

Boar taint, an unpleasant odor observed in pork from some mature intact male pigs, is attributed primarily to the presence of two compounds in boar fat, androstenone (5α-androst-16-ene-3-one) and skatole (3-methyl indole). A rapid extraction method for the preparation of fat samples for androstenone/skatole screening assays would lead to more efficient use of boar carcasses for pork production. Supercritical carbon dioxide removed 97 ± 2% of the androstenone (extraction at 40°C; 5 min) and 65 ± 3% of the skatole (extraction at 40°C; 20 min) from 0.5 g of boar backfat. Supercritical CO_2 provides the basis for a fairly rapid extraction method to remove compounds associated with boar taint.

Key Words: pork fat, supercritical-CO_2, androstenone, skatole

INTRODUCTION

BOAR TAINT, an unpleasant odor, is observed in the fat of some mature boars (intact male pigs) making their meat unacceptable for pork consumption. Several compounds have been implicated in the incidence of boar taint, but major contributors are C_{19}-Δ^{16} steroids (particularly, 5α-androst-16-ene-3-one, referred to as androstenone) and skatole (3-methyl indole) (Bonneau, 1993). These compounds accumulate in adipose tissue and their concentration in the backfat of pork carcasses is indicative of the degree of boar taint. Boars are a more economical source of pork than barrows (castrates) because of better feed efficiencies (i.e., lower feed:gain ratios) and because boars produce leaner carcasses. However, boar taint has hindered the practice of raising boars in the U.S. Five to 10% of market weight boars have unacceptable taint (Garcia-Regueiro and Diaz, 1989). Developing a screening method to sort out tainted carcasses on-line in a pork processing plant would enable more efficient utilization of boar carcasses and more efficient production of pork.

Several techniques have been suggested for measurement of boar taint, such as gas chromatography (GC) and GC/mass spectrometry (Peleran and Bories, 1985; DeBrabander and Verbeke, 1986), high-performance liquid chromatography (HPLC; Dehnhard et al., 1993), radioimmunoassays (Andresen, 1975), colorimetric tests (Mortensen and Sorensen, 1984; Squires, 1990), and a hot wire test (Jarmoluk et al., 1970). None of the tests could completely meet the requirements for time, sensitivity and accuracy. Berdague et al. (1993) subjected boar backfat to a dynamic headspace extraction method using helium. Their method was unsuccessful in extracting androstenone and skatole. Headspace volatiles did not contain any indole compounds or steroids that were detectable via GC/mass spectrometry. All analytical techniques currently used for androstenone/skatole detection rely on extraction with organic solvents—a laborious, tedious, and time-consuming procedure.

Supercritical fluid extraction (SCFE) is based on the use of gases at or above their critical temperature and pressure and is widely used for extracting various components from animal and plant materials. Solvating power of supercritical fluids (SCF) can be regulated via fluid density which is dependent on temperature and pressure. Regulating the solvating power of SCF allows the separation of extracted components from the supercritical solvent (e.g., CO_2) and is accomplished by reducing fluid pressure. No excessive sample heating or special evaporation procedures are required which is important when handling volatile compounds, such as skatole and androstenone. Our objective was to determine if supercritical carbon dioxide (CO_2) could be used to extract androstenone and skatole from pork fat.

MATERIALS & METHODS

Materials

Androstenone (5α-androst-16-ene-3-one), methoxyamine hydrochloride, and heptane (GC-grade) were obtained from Sigma Chemical Co. (St. Louis, MO). Skatole was purchased from Eastman Kodak Co. (Rochester, NY). Stock standard solutions of androstenone and skatole were prepared by dissolving them in heptane. Standard solutions were used as external standards for GC analysis and select samples were augmented with standard solutions to determine recovery. Other solvents were obtained from Mallinckrodt Specialty Chemical Co. (Paris, KY).

Supercritical CO_2 apparatus

The apparatus used was of the static type. The major component of the apparatus was a Ruska pump (Model 2200, Ruska Instrument Corp., Houston, TX) to inject CO_2 into a pressure vessel (Kuentzel Closure reactor with an internal volume ≈ 6.6 mL, Series KC single-ended unit, Autoclave Engineers, Inc., Erie, PA) at the experimental pressure. The vessel was immersed in a thermostated water bath to maintain required temperatures. The internal temperature of the vessel was not monitored. A pressure gauge was installed in the CO_2 inlet of the vessel to read pressure. The vessel and all other parts exposed to high pressure were made of stainless steel type 316.

Supercritical CO_2 extraction conditions

Extraction with supercritical CO_2 was performed using pressures of 7, 21, and 28 MPa, temperatures of 35, 40, and 45°C, and times of 5, 10, 15, 20, 25, and 30 min. In addition, some extractions were performed below the CO_2 critical point at 0°C. Samples of pork subcutaneous adipose tissue were obtained from market weight boar carcasses at a commercial packing plant. Cubed samples of fat (each 0.5g with similar height, width, and length) were placed in extraction vessels (0.5 g/vessel) and vessels were allowed to equilibrate in a water bath for 10 min. Samples were subjected to supercritical CO_2 extraction and then removed from extraction vessels. The internal surface of each cylinder was washed with organic solvent mixture (see below) which was subsequently combined with each residual sample and evaporated under vacuum. Samples were then subjected to an organic solvent extraction procedure to determine residual androstenone and skatole content. An identical sample which was not subjected to SCFE was also analyzed for initial androstenone and skatole content.

Androstenone and skatole analysis

Organic solvent extraction was accomplished using a quaternary solvent mixture (methanol/cyclohexane/benzene/water) as described by DeBrabander and Verbeke (1986). Extraction efficiencies for the organic extraction procedure were 95 ± 1% for androstenone (0.5 to 10 ppm range) and 93 ± 1% for skatole (0.2 to 1.0 ppm range). Following organic extraction, but prior to GC, extracts for androstenone analysis

were derivatized with a 2 mg/mL solution of methoxyamine hydrochloride in dry pyridine at 25°C (Singh, 1979). A Varian 3400 (GC) equipped with a thermionic specific detector (TSD) was used to quantify androstenone and skatole as described by Peleran and Bories (1985) and DeBrabander and Verbeke (1986). The column (1.83 m × 2 mm i.d.) was 3% SP-2100 on 80/100 Supelcoport (Supelco, Bellefonte, PA). Additional GC conditions were: carrier gas, nitrogen (30 mL/min); injector temperature, 240°C for androstenone and 160°C for skatole; detector temperature, 300°C for androstenone and 270°C for skatole; retention time, 9.5 min for androstenone and 5.0 min for skatole.

Data analysis

Residual androstenone and skatole in fat samples following supercritical CO_2 extraction was expressed as % of initial content. Two experiments were conducted for examining androstenone extraction with at least three samples tested/extraction condition. One experiment was conducted for skatole extraction with 1 to 7 samples extracted for each extraction condition. Data were subjected to statistical analysis using the General Linear Models program of the Statistical Analysis System (SAS Institute, Inc., 1987) with extraction pressure, temperature, and time as factors. Means were separated using Bonferroni t-tests (SAS Institute, Inc., 1987).

RESULTS & DISCUSSION

THIS STUDY focused on developing a procedure for extracting androstenone and skatole from pork fat. Androstenone and skatole detection was performed by GC using a thermionic specific detector (sensitive to nitrogen- and phosphorus-containing compounds) which increased sensitivity, relative to the non-specific flame ionization detector, and virtually removed interference from other volatile compounds present in the extract. A simple derivatization procedure was required for androstenone prior to detection. No derivatization was required for skatole, which contains nitrogen.

A major goal was to demonstrate the potential for rapid and quantitative extraction of androstenone/skatole from boar fat. Experiments on extractability from fat samples demonstrated that, under specific extraction conditions, up to 98% of the total androstenone (Fig. 1) and 65% of total skatole (Fig. 2) could be extracted from 0.5g of fat. Pressure had no significant effect (P > 0.05) on residual androstenone or skatole. Means and standard errors of residual androstenone (Fig. 1) and skatole (Fig. 2) in fat subjected to different extraction temperatures and times were compared. Lack of significant effects of pressure on extraction may have been due to the type of system we used. Our static system may limit the amount of compound extracted under given conditions. A continuous system, with CO_2 flowing through the extraction chamber may have increased extraction.

Time and temperature of extraction significantly affected extraction of both androstenone and skatole from fat. For androstenone, a time by temperature interaction existed (P < 0.07). Extractions of greater than 95% were obtained within 5 or 10 min at temperatures of 40° or 45°C. Residual androstenone was less (P < 0.05) at 40 and 45°C than at 35°C for extraction times of 5 and 10 min. Increasing times to 15 min or greater resulted in similar (P > 0.10) residual androstenone among all extraction temperatures. Thus, for androstenone, extraction time could be reduced by increasing the temperature of the extraction vessel. For skatole, temperature had no effect on extraction. At 35°C, skatole extraction was improved (P < 0.05) by increasing extraction time from 5 to 10 min, but extraction was not satisfactory at any conditions tested (>35% residual). Possibly more extreme conditions, such as higher temperatures, would improve skatole extraction. A continuous system may also improve overall extraction of skatole under the conditions tested.

When extraction vessels were maintained at 0°C, below the carbon dioxide critical point, less than 10% of the androstenone was extracted from samples at 7, 21, and 28 MPa over a 25-min period (data not shown). The supercritical state is necessary for quantitative extraction of androstenone from pork fat. Fluids
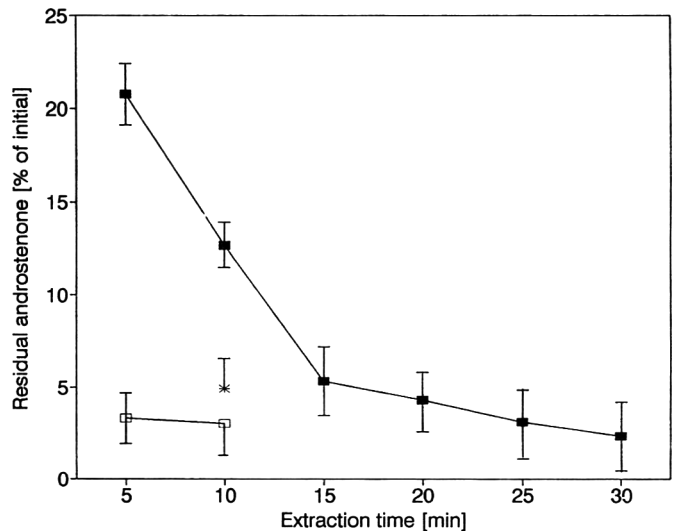
Fig. 1—Effect of supercritical CO_2 extraction temperature and time on residual androstenone in pork fat. (■) 35°C; (□) 40°C; (*) 45°C. Each point at 5 and 10 min represents the mean of 2 experiments (n≥4 samples/time). Each point at 15, 20, 25, and 30 min represents the mean of 1 experiment (n≥3 samples/time). Error bars represent ± standard errors.
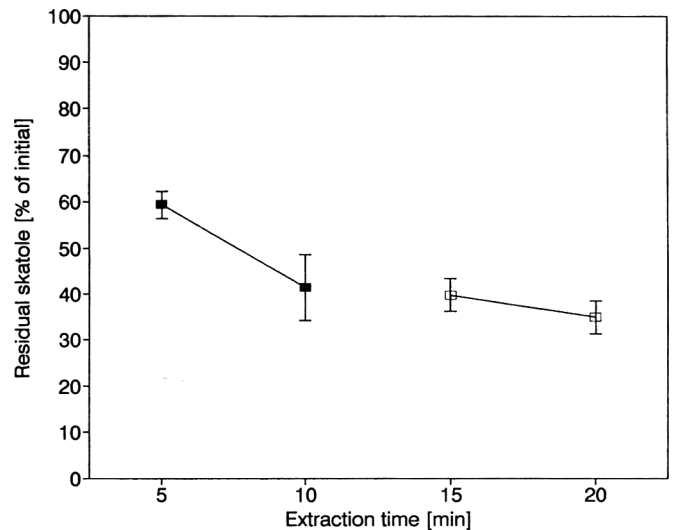
Fig. 2—Effect of supercritical CO_2 extraction temperature and time on residual skatole in pork fat. (■) 35°C; (□) 40°C. Each point represents the mean of 1 experiment (n=7, 1, 4, and 4 for 5, 10, 15, and 20 min, respectively). Error bars represent ± standard errors.

other than CO_2 may be more effective in extracting androstenone and skatole from pork fat.

Supercritical fluid extraction could be directly coupled with gas chromatographs. The use of supercritical GCs for detection and quantification of androstenone and skatole could reduce the time required for boar taint assessment in pork carcasses. From our results, combining SCFE with GC detection may provide a potential objective and rapid method of boar taint measurement. A more practical method, however, would be to use SCFE as a rapid extraction procedure prior to colorimetric assay for androstenone (Squires, 1990) and skatole (Mortensen and Sorensen, 1984). Colorimetric assays are technically feasible for on-line applications in pork processing plants. Timely organic solvent extraction procedures are used in preparing pork tissue for colorimetric assays which may not be feasible for many meat processing plants.

CONCLUSIONS

ANDROSTENONE AND SKATOLE could be extracted from pork fat using supercritical CO_2. In a static SCFE system, we obtained extraction efficiencies of 97% for androstenone and 65% for skatole. Although efficiencies for skatole were less than desired, they were reproducible at given conditions. It may be possible to correlate extracted amounts of skatole and androstenone to a level of boar taint in pork fat. These findings may lead to development of practical new methods for detecting boar taint in pork carcasses. The use of a continuous SCFE system may be a method to improve extraction and analysis time of androstenone and skatole in future studies.

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α-Tocopherol, β-Carotene and Ascorbic Acid as Antioxidants in Stored Poultry Muscle

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─────────── ABSTRACT ───────────

Broilers were fed α-tocopherol or β-carotene for 3 wk or L-ascorbic acid for 24 hr prior to slaughter. α-Tocopherol maintained the redness of unheated meat stored for 8 wk (−20°C). Values for thiobarbituric acid reactive substances in ground, stored meat showed that L-ascorbic acid produced results similar to the control while α-tocopherol produced results lower than the control. Panelists rated meat with added α-tocopherol as different from the control for smell and flavor. β-Carotene was a pro-oxidant compared to the control and other additives. Meat from broilers fed β-carotene had lower α-tocopherol content than the control. Vitamin A in livers of birds fed β-carotene was 64% higher than that of the control.

Key Words: antioxidant, α-tocopherol, β-carotene, L-ascorbic acid, stored poultry meat

INTRODUCTION

LIPID OXIDATION in raw muscle tissue is initiated in the intracellular phospholipid fractions of membranes. Changes occurring from the oxidation of lipids affect the functional, sensory and nutritive values of meat products (German, 1990). In addition, reports have shown that different by-products of lipid oxidation may be angiotoxic, carcinogenic, cytotoxic and/or mutagenic (Sevanain and Peterson, 1986; Addis and Park, 1989; Peng et al., 1991).

Several reports have suggested ways to retard lipid oxidation in poultry meat (Dawson and Gartner, 1983; Buckley and Morrissey, 1992). In many studies, polyphosphates and α-tocopherol were used as antioxidants (Ang and Hamm, 1986; King and Earl, 1988). Though somewhat effective as antioxidants, polyphosphates must be added at a point in processing after some changes caused by lipid deterioration may have already occurred (King and Earl, 1988). In contrast to polyphosphates, tocopherol can be added as a nutritional supplement which maintains antioxidant activity during processing and storage of poultry meat (Uebersax et al., 1978; Sheldon, 1984).

Results on the effectiveness of β-carotene and other oxycarotenoids as antioxidants during processing and storage of meat are not as conclusive as those for α-tocopherol (Krinsky, 1979; Terao, 1989; Leibovitz et al., 1990). Leibovitz et al. (1990) reported that nutritional administration of β-carotene at a maximum dose of 30 mg/kg of feed did not retard lipid peroxidation in rat tissue slices. However, Krinsky and Deneke (1982) used egg yolk phosphatidylcholine and synthetic DL-α-phosphatidylcholine and dipalmitoyl liposome models to show that β-carotene and canthaxanthin inhibited lipid oxidation initiated by CO_2 and other factors.

Ascorbic acid, a natural antioxidant, has been fed to poultry to inhibit lipid oxidation of stored meat. Chang (1989) added 1000 mg/L ascorbic acid to drinking water of stressed chickens for 32 hr prior to slaughter. Results from analysis of thiobarbituric acid reactive substances (TBARS) over 0 to 7 days storage

at 4°C revealed that addition of the vitamin significantly reduced lipid oxidation.

While results of individual studies report the effectiveness of single antioxidants in stored poultry meat, results of a comparative study on the efficacy of α-tocopherol, β-carotene and ascorbic acid as antioxidants in meat of healthy, unstressed poultry have not been reported. Our objective was to compare instrumental and sensory indicators of lipid oxidation in processed and stored chicken meat from birds fed excess α-tocopherol, β-carotene or L-ascorbic acid.

MATERIALS & METHODS

Reagents

Ethoxyquin (6-ethoxy-1,2-dihydro-2,2,4-trimethylquinoline), 2-thiobarbituric acid, 1,3,3-tetraethoxypropane, trichloroacetic acid, NaOH, methanol and butylated hydroxytoluene were purchased from Sigma-Aldrich NV/SA (Bornem, Belgium). Ethylenediaminetetraacetic acid (EDTA) and NaCl were from E. Merck (Darmstadt, Germany) and L-histidine monochloride monohydrate was from Kyowa, Hakko Kogyo, Co., Ltd. (Tokyo, Japan). Rovimix[R] B-Carotene (10%), Rovimix[R] E-50 SD (d-α-tocopherol acetate, 50%) and L-ascorbic acid were obtained from Roche (Mijdrecht, The Netherlands). Grade C microfiber glass filters were obtained from Whatman (Hillsboro, OR) and C_{18} solid phase extraction cartridges were obtained from Waters (Milford, MA).

Feeding broilers

Ross 1-day-old female chicks (360) were wing-banded and allotted to 24 pens of 15 birds in a randomized block (three) design (Fig. 1) with two replicates/block for four different diets (Fig. 2). Pens had wood shavings and were equipped with aluminum feeders and plastic waterers. Galvanized steel partitions were covered to avoid consumption of iron rust, a pro-oxidant in lipid oxidation. The control diet (Table 1), containing ≈80 mg/kg ethoxyquin as antioxidant, was prepared 2 wk in advance and frozen (−20°C) until fed for the first 3 wk. For the ensuing 3 wk prior to slaughter (at 6 wk of age), birds received either the control
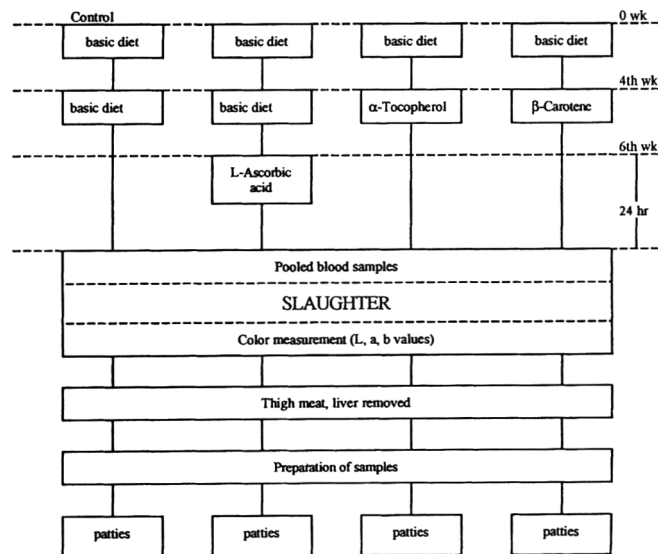

Fig. 1—Flow chart for the study.

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	Control		α-Tocopherol		β-Carotene		L-Ascorbic acid	
Block (Days of processing)	----------------------------------Replication----------------------------------							
1	1	2	1	2	1	2	1	2
2	1	2	1	2	1	2	1	2
3	1	2	1	2	1	2	1	2

Fig. 2—Experimental design for feeding and processing of birds.

Table 1—Composition of broiler control diet[a]

Feedstuffs	%
Corn	10.0
Wheat	25.0
Peas	5.8
Manioc, 65% starch	20.0
Toasted full fat soybeans	10.8
Soybean meal	14.6
Meat and bone meal, high fat	5.8
Fish meal	1.0
Animal fat	5.0
DL-methionine	0.3
Mineral premix	2.0
Vitamin premix	0.5
Zinc	0.033
Ethoxyquin	80 (mg/kg)

[a] Feed provided 3140 ME Kcal/kg, 20.3% protein, 9.1% crude fat and all requirements of the NRC (1994).

alone or the control plus an added antioxidant as follows: α-tocopherol (150 mg/kg of feed) for 3 wk, β-carotene (25 mg/kg) for 3 wk, or ascorbic acid (1500 mg/L) dissolved in tap water and administered for 24 hr prior to slaughter. For block 1, a fresh solution of ascorbic acid was made after 12 hr. Diets and water were administered *ad libitum*. Weekly weight gain and feed consumption of each replicate were recorded. The model, $Y_{ijk} = u + block_i + diet_j + e_{ijk}$ was used to statistically analyze the data. We assumed that interaction between blocks and diets could be neglected. Random error, e_{ijk}, was assumed to be independently distributed.

Blood sampling, processing of broilers and determination of color

About 4 hr prior to slaughter, blood was drawn from four birds/replicate and pooled. EDTA was used to prevent coagulation. Samples were centrifuged for 30 min at 5,000 × g to separate serum, which was stored at −80°C until analyzed. The remaining broilers (11 unbled birds/replicate) were processed at The Spelderholt, DLO Center for Poultry Research and Information Services, Beekbergen, The Netherlands. Broilers were processed by block over a 3-day period so that two replicates of each diet were processed daily (Fig. 2). Immediately after slaughter, Hunter L (lightness), a (redness) and b (yellowness) values of the femoral tract from the right thigh of each bird were determined with a standardized spectrocolorimeter (Colormet, Instrumar Ltd., St John, NF, Canada). The model delineated above with the additional parameter, e_{ijkl} (variance for birds), was used to analyze the color of femoral tract. The errors e_{ijk} and e_{ijkl} represented the errors for replicate k and bird l in replicate k in block i and diet j, respectively. Muscle with adhering fat from both thighs of broilers was placed in aluminum foil, frozen in liquid nitrogen and stored at −80°C. Livers were removed from five to six birds per replicate, pooled by block and frozen at −80°C until analyzed.

Processing of patties and determination of thiobarbituric acid reactive substances (TBARS)

Muscles were thawed for ≈1 hr, pooled by block and ground at 0°C to a grain size of about 4.5 mm diameter. Meat was stored at −80°C for later determination of antioxidants in microsomes isolated by methods of McDonald et al. (1979) and TBARS in meat after processing and grinding. The remaining meat for each block was mixed with or without NaCl (2.0% w/w) and made into patties (90 ± 0.5g, 9.0 cm in diameter) according to procedures of King and Earl (1988). Patties were individually stored in opaque plastic containers for 4 days at 4°C and at 2, 4 and 8 wk at −20°C. At the end of the storage periods, all patties were thawed for about 1.5 hr (23°C). Half of the patties with and without NaCl from all storage conditions were heated in aluminum pouches by grilling for 1.25 min on each side at 250°C. Unheated meat (after processing and grinding) and all unheated and heated patties were analyzed for TBARS values by the procedure of Raharjo et al. (1992). Duplicate determinations were made for each block per diet. Feed samples (2–3) were also analyzed for TBARS values (Raharjo et al., 1992) at the beginning and end of the experimental feeding period. For determining TBARS in meat and feed, the model, $Y_{ij} = u + diet_i + time_j + e_{ij}$ was used. We assumed there were no interactions between diet and time and that the random error e_{ij} was independently distributed.

Distribution of antioxidants

Feed samples (2–3) were analyzed for content of antioxidants (α-tocopherol and β-carotene) at the beginning and end of the experimental

feeding period. For block 1, one L-ascorbic acid solution was made at the beginning of the feeding period; another one was made after 12 hr. These solutions were analyzed at the beginning and end of each 12 hr cycle within the 24 hr feeding period to ascertain the lability of L-ascorbic acid over time. L-ascorbic acid solutions made only at the beginning of blocks 2 and 3, and samples, were analyzed for the vitamin at the beginning and end of the experimental feeding period of 24 hr. Ethoxyquin content of feed was determined at the end of the experiment by methods of AOAC (1984). α-Tocopherol and β-carotene were determined in feed, serum, microsomes, liver, and meat (after processing and grinding) by procedures of Keller (1993). Vitamin A content of liver was determined at the RIKILT Institute (Wageningen, The Netherlands). Unheated and heated patties, with and without NaCl, stored for 8 wk at −20°C were analyzed for α-tocopherol by Keller (1993). L-ascorbic acid in serum and meat (after processing and grinding) was determined by colorimetric method (Boehringer Mannheim, 1989). To analyze data for α-tocopherol and β-carotene in feed and L-ascorbic acid in solution, the statistical model for evaluation of TBARS values was used. For α-tocopherol, β-carotene and L-ascorbic acid in blood, liver, microsomes and meat (after grinding and processing), a model similar to that used for weight gain and feed consumption was used except that the error term was e_{ij} because replicates were pooled across blocks for each diet. For TBARS values in patties, data were analyzed under the split plot model as $Y_{ijklm} + block_i + diet_j + e_{ij} + NaCl_k + interaction + e_{ijk} + heating_l + interactions + e_{ijkl} + storage_m + interactions + e_{ijklm}$. The random errors from e_{ij} to e_{ijklm} represented the errors in the various strata of the design and were assumed to be independently distributed.

L (Lightness), a (redness) and b (yellowness) of meat patties

Hunter L, a and b values were determined for thawed patties with and without NaCl after processing and grinding and after storage for 8 wk at −20°C. The statistical model for TBARS values in patties was used to analyze data.

Sensory evaluation

Patties stored for 4 days at 4°C were thawed and heated as described. Heated patties with NaCl were evaluated by 15 trained panelists using a computerized, difference from reference test (de Vries, 1993). Panelists were trained female and male personnel at the Spelderholt DLO Center for Poultry Research and Information Services (Beekbergen, The Netherlands.) They ranged in age from 25–45. All panelists had previously participated in sensory evaluations to rank characteristics for chicken flavor. Panelists participated in a training session to become familiar with the type of product to be evaluated. They compared samples from each block/diet to the standard (control) for smell and flavor under red illumination. The rating scale ranged from 1 to 7 with 1 indicating minimal difference between standard and test sample, and 7 maximal difference. Analysis of variance and ranking of means for all data were performed with the SPSS/PC system (1989) for sensory evaluation.

Statistical analysis

Statistical models were developed by Vereijken (1993). Significance of means was determined using Duncan's Multiple Range Test (P < 0.05).

Table 2—Antioxidants[c] in diets and tissues of broilers

Samples[d]	Diet	Antioxidants (mg/kg) α-Tocopherol	β-Carotene	L-Ascorbic acid
Feed	Control	26.0±3.0[b]	0.4±0.3[b]	6.6±0.0[a]
	α-Tocopherol	145.0±4.0[a]	0.4±0.2[b]	6.6±0.0[a]
	β-Carotene	23.0±0.5[b]	26.0±4.0[a]	6.6±0.0[a]
Blood	Control	4.4±0.6[b]	<0.005[b]	<3.5
	α-Tocopherol	25.3±3.2[a]	<0.005[b]	<3.5
	β-Carotene	2.9±0.2[b]	0.05±0.003[a]	<3.5
	L-Ascorbic acid	4.2±0.2[b]	<0.005[b]	<3.5
Liver	Control	8.5±1.2[b]	0.11±0.14[a]	<3.5
	α-Tocopherol	105.0±9.6[a]	—	<3.5
	β-Carotene	4.1±1.6[b]	0.7±0.1[b]	<3.5
Microsome	Control	5.2±2.5[b]	<0.005	<3.5
	α-Tocopherol	13.2±5.1[a]	<0.005	<3.5
	β-Carotene	2.3±0.4[b]	<0.005	<3.5
	L-Ascorbic acid	2.0±0.3[b]	<0.005	<3.5
Meat after grinding	Control	3.6±0.4[b]	<0.005	28.7±1.5[b]
	α-Tocopherol	12.7±1.3[a]	<0.005	—
	β-Carotene	2.4±0.2[b]	<0.005	—
	L-Ascorbic acid	4.0±0.3[b]	<0.005	33.7±1.2[a]

[a,b] Means (for each antioxidant/sample) within columns with different superscripts differ (p<0.05).

[c] α-Tocopherol (150 mg/kg) or β-carotene (25 mg/kg) added to control diets of broilers for 3 wk prior to slaughter (at 6 wk of age); L-ascorbic acid (1500 mg/kg) in tap water was fed for 24 hr prior to slaughter.

[d] Samples were analyzed for each antioxidant. n=6 for α-tocopherol and β-carotene; n=3 for L-ascorbic acid; n=6 for meat after grinding.

Table 3—α-Tocopherol content of thigh meat from broilers[d] fed various diets[e]

Diets	α-tocopherol content in meat NaCl (2.0% w/w) Unheated	Heated	No NaCl Unheated	Heated
Control	1.9±0.3[b]	2.7±0.4[b]	3.8±0.53[b]	2.0±0.1[b]
α-Tocopherol	10.4±0.4[a]	12.7±0.5[a]	13.3±1.6[a]	14.1±1.4[a]
β-Carotene	0.9±0.2[c]	1.4±0.0[c]	2.7±0.2[b]	0.7±0.2[c]
L-ascorbic acid	1.4±0.3[bc]	1.5±0.5[c]	3.5±0.6[b]	1.0±0.2[c]

[a-c] Means within each column with different superscripts differ (p<0.05). n=6.

[d] Thigh meat stored in form of patties 8 wks at −20°C.

[e] Antioxidants added to control diets of broilers as α-tocopherol (150 mg/kg) or β-carotene (25 mg/kg) for 3 wk prior to slaughter at 6 wk age or L-ascorbic acid (1500 mg/kg) in tap water for 24 hr prior to slaughter.

RESULTS & DISCUSSION

THE MEAN AMOUNTS of antioxidants in feed and tissues were compared (Table 2). The 80 mg/kg ethoxyquin added to feed upon mixing decreased to 9.6 ± 0.6 mg/kg feed at the end of the study (data not shown). Monitoring of feed for content of α-tocopherol and β-carotene showed that they did not decrease over time (data not shown). Ethoxyquin was probably used to prevent lipid oxidation of feed components and added antioxidants during storage. For block 1, L-ascorbic acid solutions mixed at the beginning of each 12 hr period had a mean of 1503 ± 47 mg/kg (n = 4); after 12 hr, they had a mean value of 1308 ± 48 mg/kg (n = 4). Similar values were noted for 0 time and after 24 hr for blocks 2 and 3, where L-ascorbic acid solutions were only mixed at the beginning of each 24 hr period. Thus, L-ascorbic acid solutions did not deteriorate between 12 and 24 hr or exhibit any notable change in content from 0 to 24 hr. Dietary variation did not affect mean TBARS values of feed. Similar TBARS values (ranging from 5.5 to 6.3) have been reported for similarly stored feed samples (Barroeta et al., 1994).

Weight gain and feed efficiency for all diets and blocks were similar with overall means of 50.23 g/bird/day and 1.81, respectively. In addition, Hunter L, a and b values for femoral tract of broilers were not different. Because β-carotene is a non-pigmenting carotenoid in broilers (Nelson, 1987), it did not cause an increase in yellowness of broiler skin. Differences in color of unheated patties with and without NaCl after grinding and after 8 wk storage (−20°C) were noted by visual observa-

tion and instrumental measurement. Visual examination revealed that patties with α-tocopherol subjected to 8 wk storage maintained more red color when compared to other additives. The instrumental determination revealed that only α-tocopherol prevented oxidation of red pigments in poultry (data not shown). α-Tocopherol produced a higher value for redness of 8.37 ± 0.85 as compared to all other additives. The mean redness values for all other additives were not different from each other. The overall mean for redness of meat from the control, β-carotene and L-ascorbic acid diets was 6.42 ± 1.00. Gray (1990) reported that α-tocopherol also maintained higher redness values in pork loins as compared to controls.

Amount of α-tocopherol deposited in various tissue were compared (Table 2). When the antioxidant was fed at ≈150 mg/kg of feed, α-tocopherol content in microsomes was ≈10 times less than that reported by Asghar et al. (1990). Differences in procedures used to isolate microsomes could account for the disparity in results. α-Tocopherol concentration in the tissue was in the order: Liver > blood > meat or microsomes (Table 2). The amount of α-tocopherol found in meat immediately after processing remained constant after storage (8 wk, −20°C) and heating, even in the presence of the pro-oxidant, NaCl (Table 3). The effect of α-tocopherol as an antioxidant was verified by redness values, TBARS values (Table 4) and sensory evaluation (Table 5). Effects of α-tocopherol on TBARS values and concentration of antioxidant in tissues of poultry have been reported (Adams, 1984; Sheldon, 1984; Sheehy et al., 1994).

In contrast to α-tocopherol, β-carotene was stored in meat and microsomes in low concentrations and was not effective as an antioxidant in unheated or heated meat (Tables 2, 3, 4 and 5). This finding confirmed results of Leibovitz et al. (1990) who fed a similar amount of β-carotene to rats and found no effect. The meat we used contained only muscle and adhering fat. Maximal deposition of oxycarotenoids occurs in the shanks, skin and adipose tissue of birds. Not enough β-carotene was deposited in the membrane of muscle to reduce oxidation of polyunsaturated fatty acids of phospholipids in processed, stored meat (Tyczkowski and Hamilton, 1986). Even when ≈8.0% skin was included in the meat mixture, β-carotene was not present in high enough quantities to reduce oxidation of meat mixtures containing 14.4% fat (Barroeta et al., 1995).

Table 4—Changes in TBARS values of dark poultry meat from broilers[f] fed antioxidants as nutritional supplements

Diet[g]	TBARS values (mg malonaldehyde/kg of meat)[h] Addition of NaCl[i] no NaCl	NaCl	Heating conditions[j] Unheated	Heated	Storage[k] 5 days	2 weeks	8 weeks	Diet
Control	0.36[c]	1.30[a]	0.67[c]	1.00[a]	1.00[b]	0.75[c]	0.76[c]	0.83[a]
α-Tocopherol	0.23[c]	0.52[b]	0.25[e]	0.51[d]	1.14[e]	0.45[d]	0.55[d]	0.38[b]
β-Carotene	0.50[b]	1.37[a]	0.78[b]	1.09[a]	1.19[a]	0.82[c]	0.79[c]	0.93[a]
L-Ascorbic acid	0.46[b]	1.28[a]	0.66[c]	1.08[a]	1.01[b]	0.75[c]	0.85[c]	0.870[a]

[a-e] Means within columns and rows for main effects (addition of NaCl, heating condition, storage and overall effect of diet) with different superscripts are different (p<0.05).

[f] Slaughtered at 6 wk of age.

[g] Antioxidants added to control diet of broilers as α-tocopherol (150 mg/kg feed) or β-carotene (25 mg/kg) for 3 wks prior to slaughter or L-ascorbic acid (1500 mg/kg) in tap water for 24 hr prior to slaughter.

[h] n = 36 for main effects of addition of NaCl (2.0% w/w), heating condition; n = 24 for each storage time; n = 72 for overall effect of diet.

[i] Includes data for both heating conditions.

[j] Includes data for samples with NaCl and those without NaCl.

[k] Includes data for addition of NaCl and heating conditions.

Table 5—Sensory evaluation of poultry thigh meat[a] with NaCl (2.0% w/w)

| Diet[b] | Block | Evaluation[c] from duo-trio test | |
		Smell	Flavor
α-Tocopherol	1	18	20*
	2	23*	24*
	3	23*	26*
β-Carotene	1	13	16
	2	13	16
L-Ascorbic acid	1	17	14
	2	16	18
	3	16	14

[a] Ground meat stored 5 days at 4°C then heated for 1.25 min on each side at 250°C.

[b] Antioxidants added to control diet of broilers as α-tocopherol (150 mg/kg feed) or β-carotene (25 mg/kg) for 3 wk prior to slaughter or L-ascorbic acid (1500 mg/kg) in tap water for 24 hr prior to slaughter.

[c] 29 evaluations/block/parameter. Number with * was significant at $p \le 0.05$ and indicates number of evaluations where differences between diets (α-Tocopherol and Control, β-Carotene and Control, L-Ascorbic acid and Control) were observed.

Unheated and heated meat with NaCl and heated meat without NaCl from broilers fed β-carotene had lower quantities of α-tocopherol than the control (Table 3). As shown (Table 4) for the main effects of no heat and storage at 5 days (4°C), addition of β-carotene to samples produced higher TBARS values than all other additives. This suggested that (1) at ≤ 0.005 mg/kg (Table 2), β-carotene was a pro-oxidant in meat under certain conditions and (2) α-tocopherol counteracted the effects of β-carotene (Table 3). The latter finding confirmed results of Bendich (1990) who reported that α-tocopherol protected β-carotene from reactions by free radicals.

Livers were analyzed to determine if elevated levels of vitamin A co-existed with elevated levels of β-carotene. Amounts of vitamin A acetate in livers of broilers fed the control, or α-tocopherol supplemented feed were 181.63 mg/kg and 183.7 mg/kg, respectively. Vitamin A acetate content of birds fed β-carotene was 297.33 mg/kg which was 64% greater than that of the control. High levels of vitamin A in livers of meat animals may be toxic to humans (Bauernfield, 1980; Olson, 1983; Bendich and Langseth, 1989).

When fed to broilers at 1500 mg/L for 24 hr, deposition of L-ascorbic acid in meat increased from 28.7 mg/kg (for the control) to 33.7 mg/kg (Table 2). However, this antioxidant was not effective in retarding oxidation of meat (Tables 4 and 5). Results for the efficacy of L-ascorbic acid supplementation were in contrast to those of Chang (1989). Although there was a decrease from about 1500 to about 1300 mg/kg after 24 hr, there was ample antioxidant for test animals. We concluded that the effectiveness of L-ascorbic acid was only evident in stressed birds as reported by Chang (1989).

Barroeta et al. (1995) used 3.6 to 3.8 mg of β-carotene/kg feed and showed that the oxycarotenoid was not effective as an antioxidant in stored meat mixtures containing 2.2 to 14.4% fat. Barroeta et al. (1995) suggested that the antioxidant effect of larger supplemental amounts of β-carotene should be investigated. Our results indicate that β-carotene at 25 mg/kg in feed was not an antioxidant in poultry meat containing 13.6% fat. The small amount (<0.005 mg/kg) of β-carotene remaining in meat and microsomes was pro-oxidative. Under our conditions, L-ascorbic acid was not an effective antioxidant in poultry meat. α-Tocopherol at ≈13 mg/kg in microsomes or meat prevented lipid deterioration as indicated by lower TBARS values.

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Ms received 9/10/94; revised 4/20/95; accepted 5/7/95.

Dietary α-Linolenic Acid and Mixed Tocopherols, and Packaging Influences on Lipid Stability in Broiler Chicken Breast and Leg Muscle

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ABSTRACT

Addition of α-linolenic acid alone or plus mixed tocopherols, to the diet of broiler chickens had significant effects on composition of muscle fatty acids. Degree of unsaturation in both neutral lipids and phospholipids was increased. Both the tocopherols in the tissues and the storage stability of the meat were affected by the degree of polyunsaturation in fatty acids and dietary tocopherols. The amount of tocopherols in leg meat was higher than that of breast meat, and the antioxidant effect of dietary tocopherols was significant in cooked leg meat with hot- and cold-vacuum packaging. Dietary tocopherols were not effective in the control of lipid oxidation in loosely packaged cooked meat. Lipid oxidation in α-linolenic acid-enriched cooked broiler meat could be controlled by hot-vacuum packaging, but the antioxidant effect of hot packaging plus dietary tocopherol was greater than hot packaging or tocopherol alone.

Key Words: broiler chicken, α-linolenic acid, lipid oxidation, dietary tocopherols

INTRODUCTION

CLAIMS OF BENEFICIAL HEALTH EFFECTS of dietary ω-3 fatty acids has led to extensive research on the use of diets to enrich animal products and tissues with ω-3 fatty acids. Sklan et al. (1983) reported that feeding dietary fats affected the fatty acid composition of muscle triglycerides, but phospholipid composition was influenced only slightly. Feeding diets enriched with ω-3 fatty acids resulted in increased accumulation of ω-3 fatty acids in chicken tissue lipids and egg yolk (Phetteplace and Watkins, 1989; Olomu and Baracos, 1991; Jiang et al., 1992; Ajuyah et al., 1993). However, poultry meat enriched with ω-3 polyunsaturated fatty acids could be expected to be susceptible to lipid oxidation and flavor defects during storage (Lin et al., 1989).

Lipid oxidation is a major factor responsible for rancid flavors and other sensory defects in meat products, and is closely related to the nature, proportion, and degree of unsaturation of fatty acids. The fatty acid composition of the phospholipids of the muscle cell membranes is especially important in determining oxidative stability of meat, since oxidative changes are initiated mainly from membrane components (Pikul et al., 1984; Buckley et al., 1989). Oxidized fatty acids affect other quality characteristics of meat products such as color, texture, and nutritive value, and might be related to cancer-causing factors (Pearson and Grey, 1983; Sevanian and Peterson, 1984; Halliwell and Gutteridge, 1990).

To improve the oxidative stability of meat products, dietary supplementation of α-tocopherol (vitamin E) and its derivatives has been used for pork (Buckley et al., 1989; Lin et al., 1989; Monahan et al., 1992), turkey (Sklan et al., 1983; Sheldon, 1984) and chicken (Lin et al., 1989). Tocopherols are mainly located in cell membranes and protect membrane fatty acids and

cholesterol from peroxidative damage caused by highly reactive free radicals such as hydroxyl, peroxyl and superoxide radicals. However, the antioxidant effects of dietary tocopherol in chicken meat varied by type of muscle, and was effective only a short time after cooking (Ajuyah et al., 1993). To improve storage stability in cooked meat, Ahn et al. (1992) implemented a method to reduce oxygen contact with meat after cooking. They vacuum-packaged turkey meat patties immediately after cooking ("hot packaging") and demonstrated a reduction in extent of lipid oxidation by 50%, as compared to conventional vacuum-packaging methods. Ahn et al. (1993a) reported that prooxidants, and total lipid and fatty acid compositions of turkey patties, had effects on lipid oxidation in patties only if oxygen was freely accessible to the patties during storage. The lipid oxidation levels in raw meat before cooking were very critical in determining the extent of lipid oxidation in cooked meat (Ahn et al., 1993b).

Our objective was to determine the effects of dietary α-linolenic acid and tocopherols, and hot packaging, on fatty acid composition and lipid oxidation in cooked broiler breast and leg meat.

MATERIALS & METHODS

Feeding and meat sample preparation

One-week-old male broiler chicks (360 birds) were placed in 12 floor pens (30 birds/pen) and fed with one of three experimental diets for 5 wk. Four pens, used as replications, were allocated randomly to each of the 3 diets. The 3 experimental diets were corn-soybean meal control, 2.6% α-linolenic acid (LNA), and 2.6% α-LNA plus mixed tocopherols (200 mg/kg feed), and were isonitrogenic (21.5% crude protein) and isocaloric (3,050 kcal/kg). Full-fat flax seed was used as a source of α-linolenic acid in diets. The content of total lipids in diets was ≈ 6%. The mixed tocopherol was a mixture of beta-, delta-, and gamma-tocopherols, from Henkel Corp. (Kankakee, IL).

Breast and leg meat was hand-deboned from the 6-wk-old broiler chickens (8 birds/treatment with 2 birds/pen) after slaughter at the University of Alberta Poultry Processing Plant. Skin, tendons and visible fat were removed, and the breast or leg muscles from the same pen were pooled by hand, ground and used as one sample. From the ground breast and leg meat, about 50g were set aside for raw sample analysis, and the rest (≈ 350g for breast and 300 g for leg per each replication) formed into uniform 75g patties in a petrie dish, wrapped in aluminum foil and cooked in a pre-heated electric oven (175° C) to internal temperature about 76° C. Temperature was monitored using a probe thermometer. Immediately after cooking, two patties were vacuum-packaged ("hot packaging"), using a Multivac (type AG 500) vacuum-packaging machine, in two (for 0 day and 7 day storage) Cryovac Nylon-Polyester bags (UNIWEST, Edmonton, Alberta), at 76° C. Another patty was chilled in a refrigerator for 3 hr at 4° C and then similarly vacuum-packaged ("cold packaging"). One patty was loosely packaged in a home-use sandwich bag to provide storage with free oxygen accessibility. Samples were stored up to 7 days in a refrigerator (4° C). Total lipid, fatty acid composition and tocopherol content of raw meat, and thiobarbituric acid reactive substances (TBARS) values of raw and cooked meat samples were determined. The TBARS values of cooked meat were measured at 0 and 7 days storage at 4° C. Four replications were made for each treatment. Zero day samples were analyzed 3 hr after cooking to establish 0 time values for the hot, cold and loose packaging.

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Thiobarbituric acid analysis

TBARS values were measured by the extraction procedure of Salih et al. (1987) with some modifications. Meat (2g) was weighed into a test tube with 18 mL of 3.86% perchloric acid and homogenized with a Brinkman Polytron (Type PT 10/35, Westbury, NY) for 15 sec at high speed (set at 8-9). Butylated hydroxyanisole (BHA) was added, 0.2 mL of 3.75 mg/mL in 98% ethanol, prior to homogenization. The homogenate was filtered through Whatman #1 filter paper. The filtrate (2 mL) was mixed with 2 mL of 20 mM TBA in distilled water, and incubated at room temperature in the dark for 17 hr. The absorbance was determined at 531 nm against a blank containing 2 mL distilled water and 2 mL of 20 mM TBA solution. The TBARS numbers were expressed as milligrams malondialdehyde/kg meat.

Total fat and fatty acid composition of lipid and lipid classes

Meat (2-3g) was weighed into a test tube with 20 mL Folch solution (chloroform: methanol = 2:1, Folch et al., 1957), and homogenized with a Polytron for 5-10 sec at high speed. BHA (3.75 mg/mL) dissolved in 98% ethanol was added prior to homogenization at a level of 35 μg/mg fat. The homogenate was filtered through a Whatman #1 filter paper into a 100-mL glass-stoppered graduated cylinder, and 5 mL of a 0.88% NaCl solution was added, and mixed into it. The inside of the cylinder was washed twice with 10 mL of Folch 2 solution $(3:47:48/CHCl_3:CH_3OH:H_2O)$. After phase separation, the volume of lipid layer was recorded, and the top layer (methanol and water) of the solution was carefully siphoned off.

Organic layer (5 mL) was put in a glass scintillation vial, dried in a block heater (1 hr at 50° C) under nitrogen, and used for total fat and fatty acid composition. The dried lipids were redissolved with an appropriate amount of chloroform to provide a sample with 50 mg lipid/mL. Lipid solution (50 mL) and 0.5 mL boron trifluoride-methanol methylation solution was added into a screw-cap tube, capped tightly and incubated in a water bath at 90° C for 1 hr. After cooling to room temperature, 5 mL water and 5 mL hexane was added to the tube, the contents were mixed well, and left overnight for phase separation. The fatty acid methyl esters of lipids were separated and quantified using an automated gas chromatograph equipped with an on-column injector (Model 3400, Varian, Sunnyvale, CA) as described in detail by Jiang et al. (1992).

The rest of the organic layer from the Folch preparation was dried under nitrogen for 2 hr and redissolved with chloroform plus 2-3 drops of ethanol to provide a solution with ≈150 mg lipid/mL. The resulting sample (200 mL) was loaded onto precoated silica gel G plates (20 × 20 cm) that had been previously activated by heating at 120° C for 2 hr. The plates were developed in 2 solutions. The first solution was chloroform:methanol:water (65:25:4) and the plates were developed until the solvent front was 10 cm from the origin (about 40 min). The plates were air-dried for 10 min and then developed for about 20 min in a second tank containing hexane and diethyl ester (4:1) solution. The plates were air-dried and sprayed with 0.1% (w/v) 2'7'-dichlorofluorescein in ethanol. Triglycerides (TG), phosphatidyl ethanolamine (PE) and phosphatidyl choline (PC) were identified under UV light, scraped off into screw-cap tubes, methylated and analyzed for fatty acids as described.

Tocopherols

HPLC determination of tocopherols was by modification of the method of Zaspel and Csallany (1983). Tissue (2-3g) was weighed into a 50-mL test tube with 8 mL of acetone, 1 mL of internal standard (IS; rac-5,7-dimethyltocol, 50 mg/mL) and 5 mL of BHA (1 mg/mL), and homogenized with a Brinkman Polytron (Type PT 10/35, Westbury, NY) for 30 sec at high speed (set at 8-9). The homogenate was centrifuged at 1200 × g for 10 min and the supernatant collected. The resulting pellet was homogenized with 4 mL acetone and then centrifuged. The supernatants were pooled and dried by evaporation under nitrogen at 60° C. The dried sample was redissolved with 1 mL methanol, vortexed, centrifuged at 1200 × g for 10 min, and then the clean upper layer was collected for HPLC assay. A Supelcosil (Supelco, Bellefonte, PA) column LC18 HPLC column (150 mm × 4.6 mm) was used for the assay. The mobile phase was 50% acetonitrile and 50% methanol, run time was 8 min, and flow rate was 1.5 mL/min. A fluorescence detector (RF-535; excitation, 295 nm; emission, 330 nm) was used to monitor concentrations of tocopherols. Standards were prepared by dissolving each tocopherol standard with methanol. Concentrations were calculated as follows:

$$\text{Response factor (RF)} = \frac{(\text{mg tocopherol isomer} \times \text{area of IS})}{(\text{mg IS} \times \text{area of tocopherol isomer})}$$

$$\text{Concentration (mg/unit sample)} = \frac{(\text{mg IS} \times \text{area tocopherol isomer})}{(\text{area IS} \times \text{RF})/\text{unit sample}}$$

Statistics

The data from breast and leg meat were processed independently by the statistical analysis system (SAS Institute, Inc., 1986). Analyses of variance were calculated for dietary treatment or dietary treatments and packaging method when needed. Mean values among dietary treatments, and effects of packaging methods within dietary treatments were also compared, when applicable, by ANOVA. SNK (Student-Newman-Keuls) multiple range test was used to compare differences among mean values.

RESULTS & DISCUSSION

Fatty acid compositions of lipids, and TG, PE and PC fractions

The dietary α-linolenic acids increased the proportions of ω-3 polyunsaturated fatty acids (PUFA) and ω-6 diunsaturated fatty acids (DUFA) in lipids of breast and leg meat (Table 1), replacing monounsaturated fatty acids (MUFA) and saturated fatty acids (SAFA). However, the increase in ω-3 PUFA was much larger than that of ω-6 DUFA. For the ω-3 PUFA, α-linolenic acid (C18:3ω3) had the largest increase, while C20: 5ω3, C22:5ω3 and C22:6ω3 also had increased in both breast and leg meat as a result of dietary supplementation of linolenic acid (Table 1). Gunstone (1991) reported that when the relative autooxidation rate of fatty acid with one double bond was 1, the rates of fatty acids with 2 and 3 double bonds were 27 and 77, respectively. Therefore, the replacement of SAFA and MUFA (20% of the total lipid) by DUFA and PUFA should increase significantly the susceptibility of muscle fatty acids to lipid oxidation.

Further analysis of changes in fatty acid compositions of lipids indicated that most of the dietary α-linolenic acid (C18:3ω3) was deposited in the TG fraction and only a small portion was deposited in the phospholipids (PE and PC) (Tables 2, 3 and 4). In the TG fraction of lipids the proportion of SAFA plus MUFA was decreased by 23-28%, and those of DUFA and PUFA were increased by 6-11% and 15-17%, respectively (Table 2). In the PE and PC fractions of lipids, however, the changes were quite different from that of TG, and were smaller than that of the TG fraction. Only a small (1-2%) increase in linolenic acid in the PE fraction and even less of an increase in the PC fraction was observed. In PE, ≈ half the ω-6 PUFA and some MUFA were replaced by ω-3 PUFA, and the increases of C22:5ω3 and C22: 5ω6 were highly significant. (Table 3).

The changes in fatty acid composition of the PC fraction were even smaller than those of PE. The changes in DUFA were significant only in leg meat. Increases in ω-3 PUFA were only about 5-7% (Table 4) mainly by replacing MUFA and ω-6 PUFA. Sklan et al. (1983) reported that the fatty acid composition of muscle triglycerides was affected by dietary fat, but that of phospholipids was influenced only slightly by different diets. However, in our study, the degree of unsaturation in both PE and PC fractions was increased significantly by dietary α-linolenic acids. The increased polyunsaturation of fatty acids was a major factor in the low oxidative stability of ω-3 fatty acid enriched meat.

Tocopherols in breast and leg meat

The amounts of α-tocopherol in raw breast meat from control and α-LNA+tocopherol diets were not different than that of α-LNA diet. Gamma- and total tocopherols were highest in α-LNA+tocopherol diet. The amounts of tocopherols in cooked breast meat were similar to those in raw meat, and the highest amounts were found in α-LNA+tocopherol diet and lowest in the α-LNA diet (Table 5).

Table 1—Fatty acid composition (%) of total lipid from raw breast and leg muscles of chickens with two different dietary supplements, α-linolenic acid or α-linolenic acid plus mixed tocopherols

	Breast				Leg			
	Control	α-LNA	LNA+tocop.	SEM	Control	α-LNA	LNA+tocop.	SEM
C14:0	0.61[b]	0.44[a]	0.41[a]	0.02	0.37[b]	0.43[a]	0.58[a]	0.02
C16:0	19.46[b]	17.01[a]	16.44[a]	0.13	18.92[b]	15.02[a]	15.10[a]	0.05
C16:1ω7	4.47[b]	2.15[a]	2.34[a]	0.12	5.88[b]	3.30[a]	3.27[a]	0.03
C18:0	8.32[a]	10.06[b]	9.91[b]	0.08	6.68[a]	7.13[b]	7.46[b]	0.05
C18:1ω9	39.97[b]	26.14[a]	26.28[a]	0.22	44.17[c]	30.24[b]	28.32[a]	0.09
C18:2ω6	17.88[a]	20.85[b]	21.42[b]	0.17	18.43[a]	22.73[b]	23.43[b]	0.03
C18:3ω3	1.21[a]	10.76[b]	11.94[b]	0.25	1.76[a]	16.02[b]	16.74[c]	0.06
C20:0	0.27	0.21	0.19	0.01	0.21[b]	0.16[a]	0.15[a]	0.00
C20:1ω9	0.66[b]	0.32[a]	0.34[a]	0.03	0.63[b]	0.40[a]	0.38[a]	0.01
C20:2ω6	0.74[b]	0.67[ab]	0.60[a]	0.02	0.31	0.29	0.32	0.01
C20:3ω6	0.92	0.96	0.86	0.03	0.28[a]	0.32[b]	0.39[c]	0.01
C20:4ω6	3.15[b]	2.88[ab]	2.55[a]	0.07	1.24[b]	1.12[a]	1.22[b]	0.02
C20:5ω3	0.28[a]	1.65[c]	1.44[b]	0.03	0.09[a]	1.09[b]	0.76[b]	0.06
C22:4ω6	0.76	0.55	0.45	0.05	0.31[b]	0.15[a]	0.18[a]	0.01
C22:5ω3	0.59[a]	2.92[b]	2.69[b]	0.05	0.23[a]	0.87[b]	1.04[c]	0.01
C22:6ω3	0.72[a]	2.43[b]	2.18[b]	0.05	0.30[a]	0.77[b]	0.88[c]	0.01
Total SAFA[d]	28.67[c]	27.75[b]	26.93[a]	0.14	26.38[b]	22.70[a]	23.10[a]	0.09
Total MUFA	45.10[b]	28.60[a]	28.95[a]	0.29	50.67[c]	33.94[b]	31.97[a]	0.09
Total ω6 DUFA	18.62[a]	21.52[b]	22.02[b]	0.16	18.74[a]	23.02[b]	23.75[c]	0.03
Total ω6 PUFA	4.83[a]	4.39[ab]	3.86[b]	0.09	1.83	1.62	1.76	0.04
Total ω3 PUFA	2.80[a]	17.76[b]	18.26[b]	0.23	2.38[a]	18.72[b]	19.45[b]	0.17
ω6/ω3 ratio	8.38[b]	1.46[a]	1.42[a]	0.02	8.64[b]	1.32[a]	1.31[a]	0.03

[abc] Different letters within a row of same meat type are significantly different (p<0.05).

[d] Abbreviations: SAFA, saturated fatty acid; MUFA, monounsaturated fatty acid; DUFA, diunsaturated fatty acid; PUFA, polyunsaturated fatty acid; SEM, standard error of mean.

Table 2—Fatty acid composition (%) of the TG fraction of lipids from breast and leg muscles of chickens with two different dietary supplements, α-linolenic acid or α-linolenic acid plus mixed tocopherols

	Breast				Leg			
Fatty acid	Control	α-LNA	LNA+tocop.	SEM	Control	α-LNA	LNA+tocop.	SEM
C14:0	0.80[b]	0.00[a]	0.00[a]	0.04	1.02[c]	0.00[a]	0.59[b]	0.01
C16:0	21.42[b]	18.34[a]	17.82[a]	0.18	21.22[b]	17.48[a]	17.26[a]	0.14
C16:1ω7	4.93[b]	2.64[a]	2.57[a]	0.14	5.60[b]	3.09[a]	3.28[a]	0.04
C18:0	5.95[a]	7.12[b]	7.09[b]	0.08	5.45	6.27	5.92	0.24
C18:1ω9	46.35[b]	31.46[a]	29.63[a]	0.25	52.08[c]	31.04[b]	29.95[a]	0.20
C18:2ω6	17.15[a]	21.44[b]	22.16[b]	0.29	13.03[a]	22.71[b]	23.60[b]	0.47
C18:3ω3	1.46[a]	15.78[b]	17.91[c]	0.12	0.69[a]	16.86[b]	17.27[b]	0.20
C20:0	0.81	0.75	0.92	0.18	0.00[a]	0.31[b]	0.30[b]	0.04
C20:1ω9	0.72	0.32	0.34	0.18	0.44	0.41	0.41	0.02
C20:2ω6	0.41[a]	1.59[b]	1.24[b]	0.08	0.29	0.80	0.54	0.12
C20:5ω3	0.00[a]	0.88[b]	0.65[b]	0.08	0.19	0.63	0.53	0.08
C22:6ω3	0.00	0.00	0.00	0.00	0.00[a]	0.39[b]	0.34[b]	0.04
Total SAFA[d]	28.98[b]	26.21[a]	25.83[a]	0.28	27.68[b]	24.06[a]	24.07[a]	0.37
Total MUFA	52.00[b]	34.10[ab]	32.21[a]	0.27	58.13[b]	34.55[a]	33.65[a]	0.24
Total ω6 DUFA	17.56[a]	23.03[b]	23.40[b]	0.18	13.31[a]	23.52[b]	24.15[b]	0.29
Total ω3 PUFA	1.46[a]	16.66[b]	18.56[b]	0.16	0.87[a]	17.88[b]	18.13[b]	0.09
ω6/ω3 ratio	12.03[b]	1.38[a]	1.26[a]	0.03	15.30[b]	1.32[a]	1.33[a]	0.01

[abc] Different letters within a row of same meat type are significantly different (p<0.05).

[d] Abbreviations: SAFA, saturated fatty acid; MUFA, monounsaturated fatty acid; DUFA, diunsaturated fatty acid; PUFA, polyunsaturated fatty acid; SEM, standard error of mean.

The amounts of α-, γ- and total tocopherols in raw and cooked leg meat were highest in the α-LNA+tocopherol diet followed by control and α-LNA diets as in breast meat, but amounts of those in leg meat were about two times higher than in breast. Unlike raw breast meat, leg meat from α-LNA+tocopherol diet had detectable amounts of δ-tocopherol in both raw and cooked meat (Table 6). Pokorny (1991) reported that the antioxidant effect of tocopherol isomers was in the order δ- > γ- > β- > α-tocopherol. Although tocopherols may be considered heat-labile (Dzrezak, 1986), most tissue tocopherols (70–80%, if moisture loss in the cooked meat was accounted for) was stable under the cooking conditions we used.

TBARS values of meat

In raw breast meat, the TBARS value of the control was lower than those of the α-LNA and α-LNA+tocopherol diets whch were not different. In raw leg meat, the TBARS value of the α-LNA+tocopherol diet was lower than that of the α-LNA diet but higher than the control diet (Tables 7 and 8). The high TBARS values in raw breast and leg meat from α-LNA and α-LNA+tocopherol diets were apparently related to increased polyunsaturated fatty acids in meat lipids. The very large increase

in polyunsaturated fatty acids in the TG fraction and the smaller increase in the PE and PC fractions indicated that the fatty acid compositions of neutral lipids were also very important to the susceptibility of meat lipids to oxidation. Pikul et al. (1984) reported that phospholipids were responsible for about 90% of lipid oxidation in meat. However, the small changes in fatty acid composition of the PE and PC fractions, and the large changes in TBARS values in the meats from α-LNA and α-LNA+tocopherol diets demonstrated that the fatty acid composition of neutral lipids was also important in the oxidative stability of meat. The lower tocopherol contents in breast and leg meat from α-LNA diet compared with other dietary treatments indicated that the oxidative stress of tissues from the α-LNA diet was higher than those of other diets. The elevated tocopherols in raw meat from control and α-LNA+tocopherol diets, especially leg meat, explains the antioxidant effet of dietary tocopherols in that tissue. Although the amount of total tocopherol in breast meat from the α-LNA+tocopherol diet was two times higher than that of α-LNA, it was not high enough to provide a significant antioxidant effect.

In cooked breast meat at day 0, the TBARS values of meats with hot packaging were almost the same as those of raw meat, and the meat from the control diet had lower TBARS than those

Table 3—Fatty acid composition (%) of the PE fraction of lipids from breast and leg muscles of chickens with two different dietary supplements, α-linolenic acid or α-linolenic acid plus mixed tocopherols

Fatty acid	Breast				Leg			
	Control	α-LNA	LNA+tocop.	SEM	Control	α-LNA	LNA+tocop.	SEM
C14:0	0.80	0.63	0.60	0.05	0.51	0.81	0.75	0.15
C16:0	7.61	7.30	6.89	0.25	7.96	10.18	8.47	0.52
C16:1ω7	0.58b	0.00a	0.00a	0.07	0.48b	0.00a	0.00a	0.03
C18:0	19.21a	23.31b	23.86b	0.11	19.99	19.19	21.37	0.47
C18:1ω9	20.06b	13.71a	13.67a	0.24	16.57b	13.66a	13.25a	0.59
C18:2ω6	14.54c	10.95a	11.81b	0.15	15.61b	15.01ab	13.83a	0.24
C18:3ω3	0.47a	1.63b	2.21c	0.09	0.34a	1.80b	2.75c	0.09
C20:0	0.24b	0.38b	0.00a	0.03	0.32	0.28	0.37	0.05
C20:1ω9	0.72c	0.35b	0.00a	0.02	0.76b	0.52b	0.26a	0.04
C20:2ω6	2.00b	1.08a	0.96a	0.07	1.39b	0.50a	1.56b	0.17
C20:3ω3	0.35a	1.07b	1.15b	0.03	0.30a	0.89b	0.86b	0.01
C20:3ω6	2.27b	1.91a	1.79a	0.03	1.28b	0.77a	0.99ab	0.06
C20:4ω6	17.07b	10.65a	10.00a	0.20	20.13b	11.91a	11.31a	0.30
C20:5ω3	1.28a	5.38c	4.80b	0.06	0.93a	5.41b	5.46b	0.11
C22:1ω9	0.29b	0.18ab	0.00a	0.10	0.45c	0.28b	0.00a	0.03
C22:2ω6	0.52b	0.00a	0.00a	0.00	0.00	0.00	0.00	0.00
C22:3ω3	1.59b	0.36a	0.21a	0.02	1.54c	0.00a	0.56b	0.01
C22:4ω6	3.93b	1.12a	1.08a	0.05	4.73b	1.09a	1.05a	0.05
C22:5ω3	2.73a	9.54b	10.44b	0.19	3.07a	8.33b	8.12b	0.23
C22:6ω3	3.78a	10.29b	10.55b	0.18	3.66a	8.98b	9.05b	0.21
C24:1ω7	0.00a	0.18b	0.00a	0.01	0.00a	0.00a	0.40b	0.01
Total SAFAd	27.86a	31.61b	31.34b	0.35	28.78	30.46	30.82	0.43
Total MUFA	21.64b	14.42a	13.67a	0.31	18.25b	13.91a	14.46a	0.62
Total ω6 DUFA	16.53b	12.02a	12.76a	0.10	17.00b	15.51a	15.39a	0.16
Total ω6 PUFA	23.27b	13.68a	12.87a	0.19	26.14b	13.77a	13.35a	0.14
Total ω3 PUFA	10.69a	28.27b	29.35b	0.31	9.84a	26.36b	25.85b	0.47
ω6/ω3 ratio	3.72b	0.91a	0.87a	0.02	4.38b	1.11a	1.11a	0.01

[a,b,c] Different letters within a row of same meat type are significantly different (p<0.05).

[d] Abbreviations: SAFA, saturated fatty acid; MUFA, monounsaturated fatty acid; DUFA, diunsaturated fatty acid; PUFA, polyunsaturated fatty acid; SEM, standard error of mean.

Table 4—Fatty acid composition (%) of the PC fraction of lipids from raw breast and leg muscles of chickens with two different dietary supplements, α-linolenic acid or α-linolenic acid plus mixed tocopherols

Fatty acid	Breast				Leg			
	Control	α-LNA	LNA+tocop.	SEM	Control	α-LNA	LNA+tocop.	SEM
C14:0	0.48b	0.44b	0.36a	0.01	0.48b	0.00a	0.53b	0.03
C16:0	26.28a	28.68c	27.67b	0.13	23.94	23.22	22.83	0.26
C16:1ω7	0.85	0.43b	0.34a	0.00	0.82c	0.00a	0.26b	0.03
C18:0	10.58a	11.49b	13.09	0.05	15.99a	19.18c	18.65b	0.09
C18:1ω9	26.06c	19.78b	19.19a	0.04	23.18c	16.02a	16.74b	0.10
C18:2ω6	19.15a	20.18b	20.69c	0.07	19.54a	23.84b	23.47b	0.10
C18:3ω3	0.19a	0.70b	0.74c	0.01	0.21a	1.18b	1.10b	0.02
C20:0	0.25b	0.19b	0.11a	0.01	0.47b	0.30a	0.39ab	0.02
C20:1ω12	0.46c	0.18b	0.11a	0.08	0.38c	0.00a	0.27b	0.01
C20:2ω6	1.98c	1.04b	0.86a	0.02	1.64b	0.65a	1.48b	0.05
C20:3ω3	0.24a	0.91b	1.05c	0.01	0.25a	0.56b	0.63c	0.01
C20:3ω6	2.21	1.59	1.71	0.01	1.34a	1.47b	1.30a	0.02
C20:4ω6	6.21b	4.31a	4.55a	0.09	5.98b	5.44b	4.23a	0.13
C20:5ω3	0.42a	2.29c	1.85b	0.01	0.35a	1.69c	1.48b	0.01
C22:1ω9	0.16b	0.00a	0.00a	0.01	0.00	0.00	0.10	0.00
C22:2ω6	0.21b	0.00a	0.00a	0.01	0.00	0.00	0.00	0.00
C22:3ω3	0.47c	0.18b	0.00a	0.02	0.54b	0.00a	0.57b	0.02
C22:4ω6	1.31b	0.45a	0.44a	0.01	1.05c	0.52b	0.36a	0.01
C22:5ω3	0.92a	4.15b	4.33b	0.02	0.75a	2.90c	2.50b	0.02
C22:6ω3	1.24a	2.71b	2.70b	0.05	1.29a	2.52b	2.31b	0.05
C24:1ω7	0.35b	0.32b	0.23a	0.01	1.83b	0.51a	0.81a	0.17
Total SAFAd	37.58a	40.81b	41.22b	0.09	40.88a	42.70b	42.40b	0.17
Total MUFA	27.88c	20.71b	19.87a	0.04	26.21c	16.53a	18.17b	0.12
Total ω6 DUFA	21.34	21.21	21.55	0.06	21.17a	24.48b	24.96c	0.08
Total ω6 PUFA	9.73a	6.35a	6.70a	0.09	8.37c	7.43b	5.86a	0.04
Total ω3 PUFA	3.48a	10.94b	10.67b	0.07	3.38a	8.85b	8.62b	0.10
ω6/ω3 ratio	8.42b	2.52a	2.65a	0.02	8.77b	3.61a	3.64a	0.01

[a,b,c] Different letters within a row of same meat type are significantly different (p<0.05).

[d] Abbreviations: SAFA, saturated fatty acid; MUFA, monounsaturated fatty acid; DUFA, diunsaturated fatty acid; PUFA, polyunsaturated fatty acid; SEM, standard error of mean.

of α-LNA enriched diets. The TBARS values of cold-packaged breast meat from all 3 diets were higher than those of hot-packaged ones. The TBARS values of cold-packaged control diet breast meat were lower than those of α-LNA and α-LNA+tocopherol diets, but no difference was observed between α-LNA and α-LNA+tocopherol treatments. The TBARS values of cold-packaged breast meat from all 3 dietary treatments were higher than those of hot-packaged ones, and the control meat had lower TBARS values than the other meats. During the 3-hr chilling period, the TBARS values of cold-packaged meat with highly polyunsaturated fatty acids increased much faster than

those of the control, and the dietary tocopherols did not have much effect on the oxidation of breast meat at day 0.

In cooked leg meat at day 0, antioxidant effects of tocopherols were also observed in all packaging methods. TBARS value of hot-packaged leg meat from the α-LNA+tocopherol diet was as low as that of control, but leg meat from both control and α-LNA+tocopherol diets were lower in TBARS than that of the α-LNA diet. The TBARS value of cold-packaged leg meat from the α-LNA+tocopherol diet was lower than that of the α-LNA diet, due to the antioxidant effect of extra tocopherols incorporated in the meat, but still was higher than that of the control.

Table 5—Effect of two dietary supplements (α-linolenic acid or α-linolenic acid plus mixed tocopherols) on the relative tocopherol content of raw and cooked chicken breast muscle[d]

Dietary treatment	Tocopherols				Tocopherols			
	alpha-	gamma-	delta-	total	alpha-	gamma-	delta-	total
	Raw meat				Cooked meat			
Control	1.74[b]	0.89[b]	0	2.63[b]	1.6·[a]	0.84[b]	0	2.45[b]
α-LNA	0.90[a]	0.59[a]	0	1.49[a]	0.90[c]	0.62[a]	0	1.52[a]
α-LNA+tocopherol	1.95[b]	1.09[c]	0	3.04[c]	2.04[c]	1.27[c]	0.07	3.38[c]
SEM[e]	0.05	0.02	0.00	0.05	0.04	0.02	0.00	0.05

[abc] Different letters within a column are significantly different (p<0.05).
[d] 0 day samples were used for the cooked meat tocopherols.
[e] Abbreviations: SEM, standard error of mean.

Table 6—Effect of two dietary supplements (α-linolenic acid or α-linolenic acid plus mixed tocopherols) on the relative tocopherol content of raw and cooked chicken leg meat[d]

Dietary treatment	Tocopherols				Tocopherols			
	alpha-	gamma-	delta-	total	alpha-	gamma-	delta-	total
	Raw meat				Cooked meat			
Control	2.32[b]	1.74[b]	0	4.06[b]	2.66[b]	1.84[b]	0	4.50[b]
α-LNA	1.62[a]	1.36[a]	0	2.98[a]	1.58[a]	1.32[a]	0	2.90[a]
α-LNA+tocopherol	2.82[c]	2.32[c]	0.30	5.44[c]	3.10[b]	3.01[c]	0.27	6.38[c]
SEM[e]	0.05	0.04	0.01	0.08	0.11	0.05	0.01	0.13

[abc] Different letters within a column are significantly different (p<0.05).
[d] 0 day samples were used for the cooked meat tocopherols.
[e] Abbreviations: SEM, standard error of mean.

Table 7—Effect of two dietary supplements (α-linolenic acid or α-linolenic acid plus mixed tocopherols) on TBARS values of raw and cooked chicken breast meat with different packaging methods during storage

Dietary treatment	Raw	Cooked meat					
		0 day[d]			7 day		
		Hot pkg	Cold pkg	Loose pkg	Hot pkg	Cold pkg	Loose pkg
		TBARS[e] values (mg malondialdehyde/kg meat)					
Control	0.16[a]	0.17[ax]	0.50[ay]	0.50[ay]	0.20[ax]	0.61[ay]	3.21[az]
α-LNA	0.61[b]	0.73[bx]	1.87[by]	1.87[by]	0.89[bx]	2.35[by]	6.32[cz]
α-LNA+tocopherol	0.67[b]	0.73[bx]	1.79[by]	1.79[by]	0.93[bx]	2.76[by]	5.60[bz]
SEM[e]	0.01	0.01	0.03	0.03	0.02	0.06	0.07

[abc] Different letters within a column are significantly different (p<0.05).
[d] 0 day samples were analyzed 3 hr after cooking.
[e] Abbreviations: TBARS, 2-thiobarbituric acid reactive substances; SEM, standard error of mean.
[xyz] Different letters within a row of same storage period are significantly different (p<0.05).

Table 8—Effect of two dietary supplements (α-linolenic acid or α-linolenic acid plus mixed tocopherols) on TBARS values of raw and cooked chicken leg meat with different packaging methods during storage

Dietary treatment	Raw	Cooked meat					
		0 day[d]			7 day		
		Hot pkg	Cold pkg	Loose pkg	Hot pkg	Cold pkg	Loose pkg
		TBARS[e] values (mg malondialdehyde/kg meat)					
Control	0.19[ax]	0.21[ax]	0.83[ay]	0.83[ay]	0.23[ax]	1.01[ay]	5.56[az]
α-LNA	0.66[cx]	0.87[bx]	2.58[cy]	2.58[cy]	1.01[bx]	3.08[cy]	9.32[cz]
α-LNA+tocopherol	0.37[by]	0.23[ax]	1.01[bz]	1.01[bz]	0.52[ax]	1.84[by]	8.35[bz]
SEM[e]	0.01	0.01	0.02	0.02	0.03	0.05	0.12

[abc] Different letters within a column are significantly different (p<0.05).
[d] 0 day samples were analyzed 3 hr after cooking.
[e] Abbreviations: TBARS, 2-thiobarbituric acid reactive substances; SEM, standard error of mean.
[xyz] Different letters within a row of same storage period are significantly different (p<0.05).

After 7 days storage in the cold room (4° C), the increase in TBARS of hot packaged breast meat was as low as 0.2, too low to indicate any oxidized rancid flavor. In cold-packaged breast meat, the increase of TBARS values was much larger than those of hot-packaged and the values were high enough to indicate an oxidized rancid flavor. Again, the addition of tocopherols to the α-LNA (α-LNA+tocopherols) diet had no antioxidant effect in breast meat with hot and cold packaging after 7 days storage. In loosely packaged breast meat, however, the dietary tocopherols had some antioxidant effect, and the TBARS values of breast meat after 7 days storage were 3-5 times higher than those of 0 day.

After 7 days storage, antioxidant effects of tocopherols were found in cooked leg meat with all three packaging methods. However, the effects of tocopherols in loosely packaged leg meat was not enough to completely prevent lipid oxidation. The use of hot packaging with the leg meat from the α-LNA+tocopherol diet was much more effective than that from the α-LNA diet, partly due to the low initial TBARS from the α-LNA+tocopherol diets which after 7 days storage were still low. The antioxidant effect of dietary tocopherols was stronger than the reported effects of tocopherols added to meat before processing (Ahn et al., 1993b). Many researchers have indicated that dietary supplementation of tocopherols could improve the storage stability of poultry, pork, beef and veal (Sklan et al., 1983; Sheldon, 1984; Lin et al., 1989; Faustman et al., 1989;

Monahan et al., 1992; Engeseth et al., 1993). Buckley et al. (1989) reported enhanced stability of pork muscle lipids by dietary supplementation with α-tocopherol.

Except for the α-LNA + tocopherol treatment, where tocopherol was an effective antioxidant, the lipid oxidation in cooked leg meat was more pronounced than that in cooked breast meat. This was due mainly to the high fat content in leg, but the differences were not as great proportionally as differences in total lipids (1.5% breast, 5.7% leg). This indicated that the amounts of total lipid as well as composition of fatty acid of lipids and phospholipids were both important in lipid oxidation of meat. The contribution of phospholipids to the oxidation of meat may not be as great as has been reported (Pikul et al., 1984).

CONCLUSION

DIETARY α-LINOLENIC ACID can effectively enrich broiler muscles with ω-3 PUFA, and dietary tocopherols increased the amounts of tocopherols in tissues. However, tocopherols in breast muscle was not high enough to control lipid oxidation. The lipid oxidation of ω-3 PUFA-enriched broiler meat could be controlled effectively by hot-vacuum packaging, alone or with dietary tocopherol.

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Cross-reactivity and Heat Lability of Antigenic Determinants of Duck and Goose Lysozymes

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ABSTRACT

Two panels of monoclonal antibodies (Mabs) raised against duck (Barbary) egg white lysozyme or hen egg white lysozyme, were tested in antigen-coated plate (ACP) and double antibody sandwich (DAS) ELISA for cross-reaction with various avian lysozymes. The antibodies to hen lysozyme cross-reacted with goose lysozyme, but most antibodies to duck lysozyme reacted with it. One antibody to duck lysozyme reacted more strongly with goose lysozyme than with the homologous antigen, a heterospecific reaction confirmed by biosensor technology. Many lysozyme epitopes recognized by different antibodies showed considerable resistance to heat denaturation. Such Mabs may be useful for detecting chicken liver adulterants in "foies gras" of goose or duck origin.

Key Words: monoclonal-antibodies, duck, goose, lysozymes, antigen

INTRODUCTION

"Foie gras" is a very high value, expensive food made of goose or duck liver in which adulterants such as chicken or turkey liver or egg-whites are illegal by French regulatory authorities. However, for technological or economic reasons, such adulterants are sometimes used and techniques are needed for detecting and quantifying them. We have studied lysozyme as target protein for revealing the presence of adulterants because it is present in eggs, liver and blood and is easily extracted.

Chicken hen egg white lysozyme (HEWL) has been used extensively as a model protein to study the molecular basis of enzymatic activity (Chipman and Sharon, 1969) and antigenic specificity (Prager and Wilson, 1971; Atassi and Lee, 1978a and b; Ibrahimi et al., 1980; Smith-Gill et al., 1982; Harper et al., 1987; Lavoie et al., 1989). Primary sequences of lysozymes have also been compared to analyze protein evolution (Jollès and Jollès, 1984). There is considerable sequence homology among the c-type (chicken, duck) lysozymes, but almost no homology between c-type and g-type (goose) lysozymes, although they exhibit similar enzymatic activity (Schoentgen et al., 1982). When antigenic relationships between these two types of lysozymes were analyzed by immunoprecipitation and complement fixation, no antigenic cross-reaction between them was detected (Arnheim and Steller, 1970; Prager and Wilson, 1974; Jollès and Jollès, 1984).

In contrast, ELISA tests with polyclonal antibodies showed that c-type and g-type lysozymes cross-reacted antigenically (Hemmen et al., 1992). It seemed likely that, by using specific Mabs to differentiate between proteins of different species, it should be possible to detect adulterants in goose and duck "foie gras".

In a previous study (Hemmen et al., 1993), the ability of Mabs raised against HEWL or DEWL to react with pure duck and goose "foie gras," duck and goose "foie gras" mixed with fresh chicken liver, fresh turkey liver or chicken egg white was determined. Some anti-HEWL Mabs reacted with lysozyme present in duck and goose livers. No anti-duck egg white lysozyme (DEWL) Mabs reacted with pure duck "foie gras"

whereas some of them cross-reacted with HEWL. We concluded that duck and goose lysozymes present in liver were different from those present in duck or goose egg white. Anti-DEWL Mabs should be useful reagents to detect egg whites or chicken liver in "foie gras" since they exhibited no reaction with pure "foie gras" of goose or duck origin. These unexpected results emphasized the need to examine the cross-reactive potential of anti-DEWL and anti-HEWL Mabs to some lysozymes. In addition, when "foie gras" was heated at 80°C, a few anti-HEWL Mabs exhibited a positive reaction when used for detecting the antigen in 5 mg/mL of liver proteins. To develop a suitable method for detecting various lysozymes in food, it was necessary to analyze the cross-reactive potential of different anti-lysozyme Mabs. Different immunoassays were tested in order to select the most sensitive ones and the thermosensitivity of the relevant epitopes was determined.

MATERIALS & METHODS

Lysozymes

HEWL was purchased from Sigma Chemical Co. (St. Louis, MO). GEWL (Embden goose) was generously provided by Dr. P. Jollès (Dianoux and Jollès, 1967). DEWL-Cm (Carina moschata, also called Barbary duck) was purified as previously described (Hemmen et al., 1992). DEWL-Ap (Anas platyrhynchos) belongs to the Kaki DEWL type (Jollès and Jollès, 1984) whose sequence exhibits important homology with hen lysozyme. In order to study their thermal denaturation, lysozymes (100 µg/mL) were heated at either 60°C or 85°C in a water bath or at 100°C for 30 min in an autoclave.

Antibody preparation

Polyclonal sera. Six-month-old female White New Zealand rabbits were given an intradermal injection of 100 µg of either HEWL, DEWL-Cm, DEWL-Ap or GEWL in complete Freund's adjuvant and sera were harvested 15 days later. The rabbits received four intradermal boosts, each of 100 µg of antigen, in incomplete Freund's adjuvant at monthly intervals and sera were collected 5, 10, and 15 days after each boost. Pooled sera were stored at 4°C after addition of 0.02% sodium azide. For each antigen, two rabbits were used.

Monoclonal antibodies (Mabs). The anti-HEWL Mabs have been described previously (Smith-Gill et al., 1982) and were kindly provided by Dr. S. Smith-Gill (Bethesda, Maryland). Mabs were produced against DEWL-Cm only. One 6 wk-old female BALB/c mouse was immunized following the same protocol used for production of polyclonal sera but only 10 µg of DEWL was injected each time. Spleen cells were harvested on day 4 after the third booster injection. Anti-DEWL hybridomas were obtained by fusing spleen cells of the immunized BALB/c mouse to azaguanine-resistant X63 cells (Kearney et al., 1979) with a protocol slightly modified from that described by Köhler and Milstein (1975). After 10 to 15 days, the supernatants of wells containing cells were tested for anti-lysozyme (DEWL, HEWL and GEWL) antibodies by ACP-ELISA tests. Positive clones were recloned at least twice. Seven Mabs were obtained. Anti-DEWL Mabs were concentrated by ammonium sulfate precipitation from ascites and stored at −20°C.

Enzyme-linked immunosorbent assay (ELISA)

Antigen-coated plate (ACP) ELISA. The method described by Dore et al. (1987) was used. Plates were coated with lysozyme at 1 µg/mL in carbonate buffer, pH 9.6. After rinsing, the plates were incubated for 1 hr with 5% fat-free milk solution in carbonate buffer. After washing, dilutions of Mabs in phosphate-buffered saline, pH 7.4, containing 0.05% Tween 20 (PBST) were added for 2 hr. After further washing, anti-mouse sheep immunoglobulins conjugated with alkaline phosphatase diluted 1/3000 in PBST were added for 2 hr. After rinsing, the substrate p-nitrophenyl phosphate (1 mg/mL in 0.1M diethanolamine

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Table 1—Reactivity of anti-DEWL-Cm Mabs with different lysozymes in ELISA and with BIAcore[a]

Mabs anti DEWL-Cm	Test	Antigens			
		DEWL-Cm	DEWL-Ap	GEWL	HEWL
B1H1	ACP ELISA	2.8	0.1	2.2	0.1
	DAS ELISA	1.4	0.1	0.4	nd[b]
	RU (BIAcore)	97	35	55	10
D5D6	ACP ELISA	2.4	0.4	2.0	0.05
	DAS ELISA	1.0	0.2	0.3	nd
	RU (BIAcore)	130	41	62	6
A1C1	ACP ELISA	1.1	2.9	0.05	0.1
	DAS ELISA	0.8	2.4	0.5	nd
	RU (BIAcore)	254	302	106	143
D4E2	ACP ELISA	0.1	0.1	0.5	0.1
	DAS ELISA	0.1	0.1	0.3	nd
	RU (BIAcore)	27	23	107	14

[a] ELISA values correspond to an average of OD values read in six wells with a standard deviation comprised between 0.045 and 0.095. Each BIAcore experiments was performed three times, the RU responses obtained in the three runs were very similar (the standard deviation was 15 RU).

[b] nd = not determined.

buffer, pH 9.8) was added for 40 min and the absorbance read at 405 nm.

Double-antibody sandwich (DAS) ELISA. Two formats of this assay were used. In one format (Table 2), plates were coated with either anti-HEWL or anti-DEWL Mabs (1 and 5 µg/mL, respectively) in carbonate buffer for 2 hr at 37°C or overnight at 4°C. After washing and blocking by a 5% fat-free milk solution in carbonate buffer, different lysozyme concentrations diluted in PBST were added overnight at 4°C or 2 hr at 37°C. Homologous rabbit sera diluted 1/3000 in PBST were then added for 2 hr. The plates were washed again and incubated with goat anti-rabbit IgG coupled to horseradish peroxidase (1/3000 in PBST, BIORAD) for 1 hr at 37°C. The final reaction was visualized by addition of 3, 3', 5, 5'-tetramethyl-benzidine in the presence of H_2O_2 for 15 min at 37°C. The reaction was stopped by addition of HCl (final concentration 0.25 M) and absorbance read at 450 nm. In the second DAS-ELISA format, homologous rabbit antibodies (1/3000 dilution) were adsorbed on the plates by the usual coating procedure. The blocking step and lysozyme incubation were performed as in the first DAS ELISA format. Mabs were added followed by sheep anti-mouse globulin conjugate labelled with alkaline phosphatase and its substrate as described.

Biosensor measurements

An automated optical biosensor instrument (BIAcore™ Pharmacia Biosensor AB, Uppsala, Sweden) was used to measure antibody reactivity (Malmqvist, 1993). The BIAcore instrument allows a quantitative analysis of molecular interactions in real time. This instrument uses surface plasmon resonance, a quantum mechanical factor that enables the detection of changes in optical properties at the surface of a thin gold film placed on a glass support (sensor chip). As a result a sharp dip in intensity of reflected light is produced at a specific angle. The position of the resonance angle depends on the refractive index which is directly correlated to the concentration of material in the medium. The system can then detect the binding between a ligand immobilized on the sensor chip and an analyte introduced in a flow passing over the surface. The resonance angle is monitored continuously, so that association or dis-

sociation of molecules from the sensor surface can be followed in real time. Changes in the concentration of molecules are expressed in resonance units (RU). A carboxylated dextran layer is attached to the gold surface, so that interactions occur in this hydrophilic matrix. The carboxyl groups are activated to N-hydroxysuccinimide esters, allowing immobilization of most proteins through primary amines. The stable binding enables regeneration of the sensor surface with acidic or basic solutions. The BIAcore™ system also includes a miniaturized cartridge which controls delivery of samples and reagents to the sensor chip, an autoinjector and software for system control and evaluation of results. Mabs were immobilized on sensor chips via covalently bound rabbit anti-mouse globulins (Fägerstam et al., 1990). A volume of 10 µL of Mab (ascitic fluid diluted in Hepes buffer) was injected. Then the surface was washed with HBS pH 7.4 (10 mM Hepes, 150 mM NaCl, 3.4 mM EDTA, 0.05% Surfactant P20) for 10 min to remove free Mab. Ten µL of lysozyme was injected. The threshold for significant binding was 10 resonance units (RU). The biosensor technique has been described in detail (Fägerstam and Karlsson, 1994).

RESULTS

MOST MABS TO DEWL reacted with the homologous antigen more strongly in ACP than in DAS-ELISA, while in the case of anti-HEWL Mabs the reverse pattern was observed. Unexpectedly the anti-DEWL antibodies differentiated between DEWL-Ap and DEWL-Cm (Table 1). Some antibodies reacted more strongly with the homologous DEWL-Cm (Mabs B1H1 and D5D6) while one (A1C1) reacted more strongly with DEWL-Ap.

The ability of four of the Mabs to DEWL-Cm to cross-react with other lysozymes in the two ELISA formats and in the BIAcore was analyzed (Table 1). These antibodies cross-reacted to various degrees with GEWL, and in one case (Mab D4E2) the reaction with GEWL was stronger than with either of the two DEWL. The heterospecific nature of Mab D4E2 was revealed in both ELISA formats and was confirmed with the BIAcore. The reactivity of this antibody with the two DEWLs in ELISA was at the limit of detectability.

In ACP and DAS-ELISA the anti-HEWL Mabs reacted with DEWL but not with GEWL (results not shown). Thus, while immunization with DEWL induced antibodies that usually cross-reacted strongly with GEWL and sometimes weakly with HEWL. Immunization with HEWL induced antibodies that cross-reacted with DEWL but not with GEWL.

The thermal sensitivity of lysozymes was studied by comparing concentrations of heat-denatured soluble antigens and native soluble antigens required to reach the same optical density in DAS-ELISA. The anti-HEWL Mabs (Table 2) recognized thermosensitive epitopes of HEWL that were denatured either at 60°C (Mabs HyHEL-9, 15, 501) or at 85°C (Mabs HyHEL-7, 8, 10). One Mab (HyHEL-5) was specific for a thermoresistant epitope that was denatured only at 100°C. The epitopes of DEWL recognized by these Mabs appeared to be more heat-

Table 2—Heat resistance of lysozyme epitopes measured by DAS ELISA

Mabs	HEWL				DEWL-Ap				GEWL			
	Native	60°C	85°C	100°C	Native	60°C	85°C	100°C	Native	60°C	85°C	100°C
anti-HEWL												
HyHEL-5	1[a]	1	1	100	70	70	70	600	—[b]	—	—	—
HyHEL-7	1	1	60	300	100	100	100	1000	—	—	—	—
HyHEL-8	1	2	10	700	1	1	1	60	—	—	—	—
HyHEL-9	3	10	600	600	1	1	1	10	—	—	—	—
HyHEL-10	1	1	20	300	60	60	60	300	—	—	—	—
HyHEL-15	5	10	10	300	10	10	10	100	—	—	—	—
HyHEL-501	5	20	60	300	200	200	200	600	—	—	—	—
anti-DEWL-Cm												
4A1C1	—	—	—	—	30	100	200	600	—	—	—	—
10D5D6	—	—	—	—	—	—	—	—	3	3	3	60
11B1H1	—	—	—	—	—	—	—	—	2	2	2	70

[a] Values represent antigen concentrations (ng/mL) required to give an OD_{405} - 0.5 in DAS ELISA. The Mabs were used as capture antibodies. The trapped antigens were detected with homologous rabbit antisera followed by incubation with goat anti-rabbit IgG coupled to horseradish peroxidase and its substrate.

[b] (—) Negative.

resistant than those of HEWL, and three antibodies (HyHEL-8, 9, and 15) reacted strongly with DEWL heated at 100°C.

DISCUSSION

MOST MABS TO DEWL reacted more strongly with the various lysozymes in ACP-ELISA than in DAS-ELISA. This difference was not due to a general low reactivity of antibodies used in DAS-ELISA since the level of response did not increase when less diluted antiserum was used in the DAS assay (results not shown). Apparently these Mabs found preferentially to epitopes that has been altered when the lysozymes were adsorbed to the microtiter wells. In contrast, the anti-HEWL antibodies reacted more strongly in DAS-ELISA than in ACP-ELISA, indicating that they preferentially recognized epitopes on the native lysozyme that were no longer present on the molecules after adsorption to plastic. Alterations in antigenic properties of proteins following adsorption to plastic in ELISA have been well documented for many antigens (Butler, 1991; Darst et al., 1988; Friguet et al., 1984; Smith and Wilson, 1986).

Most anti-DEWL Mabs reacted strongly with GEWL while the anti-HEWL Mabs did not. Several common epitopes (detected by mabs B1H1, D5D6, A1C1) are present in duck lysozyme (c-type) and goose lysozyme (g-type), although it has been generally proposed that c-type lysozymes do not cross-react with g-type lysozymes. This probably resulted from the fact that cross-reactions between chicken and goose lysozymes were considered but not those between duck and goose lysozymes. We found one Mab (A1C1) that could bind duck, goose and chicken lysozymes.

In our study, the specificity of the antibodies was verified with a biosensor technology which avoids the type of denaturation which may be encountered when protein antigens are adsorbed to plastic in ELISA. In the BIAcore, Mabs are trapped on a carboxylated dextran matrix by a first layer of rabbit anti-mouse globulins and are tested for their ability to recognize and capture the free lysozyme. In such assay, both Mabs and antigen are not adsorbed on a solid phase and are therefore likely to keep their native conformation. Moreover, the BIAcore makes it possible to visualize each successive binding step which is not the case in ELISA, and complications arising from nonspecific binding to the solid phase can be eliminated. Mab A1C1 found to GEWL and HEWL in the BIAcore, although it was not active in ACP ELISA nor in DAS ELISA when this Mab was used as capture antibody. The adsorption of either Mab A1C1, HEWL or GEWL to the plastic surface would clearly lessen their reactivity.

The heat resistance of different lysozymes was examined to determine any relationships between lysozyme sequence and heat lability (Malcolm et al., 1990). The study of heat-resistant epitopes is of particular interest in food analysis. Reagents specific for such epitopes could be used for detecting fraudulent additives in processed foods (Haydn, 1981; Kang'ethe and Lindqvist, 1987).

In contrast to the results of Kenett et al. (1990), we observed that by heating c-type lysozyme for 30 min at different temperatures (60°C, 85°C and 100°C) we could detect a few thermo-resistant epitopes in HEWL, although most epitopes were thermosensitive with antigenic changes occurring between 60°C and 85°C (Table 2). DEWL was more heat-resistant than HEWL since in most cases structural changes occurred only between 85°C and 100°C. Differences in heat lability of different epitopes could not be detected by ACP ELISA (data not shown) and were only revealed by a sandwich ELISA in which Mabs were used to capture the antigen.

By using Mabs to chicken or duck lysozymes as described, it should be possible to detect additives such as egg whites or fresh livers from different species (chicken, duck and goose) in various foods and to detect chicken liver adulterants in goose and duck "foie gras," as shown previously (Hemmen et al., 1993).

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Ms received 10/17/94; revised 4/15/95; accepted 5/12/95.

Poultry Processing Line Speeds as Related to Bacteriologic Profile of Broiler Carcasses

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─────────── ABSTRACT ───────────

Aerobic plate counts, Enterobacteriaceae, *E. coli* counts; and *Salmonella* prevalence were determined on broiler carcasses obtained in a commercial slaughter establishment in Puerto Rico. Samples were obtained from three sites in the production process. Each site represented an end-point of a discrete segment of the slaughter process. Whole bird carcass rinses (300) were randomly collected over 4 days of production for several linespeeds. Samples were collected for birds processed at 70 birds/min (BPM), 80 BPM, 90 BPM, and three replications of samples at 100 BPM (June 1990 to May 1991). A total of 1,800 carcass rinses were analyzed. Mean bacterial counts and *Salmonella* prevalence did not change significantly with processing line speeds.

Key Words: poultry, line speed, microbiology, salmonella, broiler carcasses.

INTRODUCTION

U.S. DIETS have greatly increased in amounts of poultry consumed. During 1990 per capita consumption of young chicken exceeded beef. By 1994 the retail weight, per capita consumption was young chicken 69.8, turkey 18.0, beef 67.5, veal 1.0 pounds, lamb/mutton 1.2, and pork 53.1, (Witucki, 1995).

Poultry production has expanded sharply to meet growing markets. The number of federally-inspected broilers increased from 1.7 billion in 1961 (Agriculture Statistics, 1962), to 6.6 billion in 1993 (Statistical Summary, 1994). Processing plants gradually reduced their work force and added automated machinery, leading to automatic venting and evisceration equipment. Genetic poultry improvements reduced growing time, eliminated sex size differences and increased disease resistance. The number of broilers condemned in U.S. inspected establishments dropped from 6% in 1961 to slightly less than 1% in 1993 (Agricultural Statistics, 1962; Statistical Summary 1994). Infectious diseases were minimized by more effective vaccines.

The industry was required to increase slaughter production rates to meet market demands. Slaughter line speeds in the 1970's were 23 birds per minute (BPM) per inspector. Usual slaughter line configurations with two Food Safety and Inspection Service (FSIS) inspectors permitted production of 46 BPM.

FSIS developed a modified traditional inspection (MTI) system for broilers in the late 1970s. The standard MTI processing line had three FSIS inspectors. The first inspector examined the outside, the line split and the next two evaluated viscera, the birds interiors, and made final carcass disposition. This system allowed processing at 70 BPM. By the early 1980s, increasing production increases in demand for poultry products, declining evidence of pathology and improved processing equipment led to investigations of increasing line speeds.

The New Line Speed System (NELS) was implemented in 1984 to allow inspection at increased line speeds. NELS allows slaughter line speeds of 91 BPM with three FSIS inspectors.

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FSIS inspection is in conjunction with a plant-developed partial quality control (PQC) program. Such programs require plants to establish process control of their operations, set standards for critical procedures, and monitor the operations to ensure they meet standards. This system allows inspectors to concentrate on conditions affecting final disposition of the birds and makes processors' responsible for quality defects such as breast blisters, bruises, and fractures. There has been concern that such processing innovations may be responsible for increased incidence of human enteric disease as a result of inspections at faster line speeds. If line speeds were reduced, it has been claimed the incidence of human enteric disease may be sharply reduced or eliminated (Bronstein, 1991; Bruce, 1990; Holson, 1992; Ingersoll, 1990). No reports have been published evaluating any correlation, between processing line speeds and microbiological contamination levels on ready-to-cook broilers.

Our objective was to determine of there was any correlation between increased line speeds and bacterial contamination of broilers. A commercial USDA inspected poultry plant in Puerto Rico was used as a source of data to quantify bacterial loads on broiler carcasses at various line speeds. The plant had been used for previous studies providing microbiological baseline data of processing operations (James et al., 1992a).

Poultry husbandry, hatching egg sources, hatchery, grower management, feed production, and all aspects of processing were similar to previous studies at this plant (James et al. 1992a, b, c).

MATERIALS & METHODS

CARCASS RINSE SAMPLES from young chickens, <7 wk old were collected during 1990 and 1991. Whole-carcass rinse samples were obtained from three sites in the production process, each representing an end-point of a discrete segment of the slaughter process.

Site 1—Preevisceration. Chicken carcasses had been processed through hanging, stunning, bleeding, scalding, defeathering, singeing, rinsing, and feet removal.

Site 2—Prechill. Carcasses had been vented, eviscerated, and USDA inspected. Necks, lungs, and viscera had been removed, and carcasses were trimmed as required and rinsed twice with 20ppm chlorinated water before arriving at this location. One rinse was done by an inside/outside body washer.

Site 3—Postchill. Chicken carcasses were chilled to at least 4.5°C after ≈ 1 hr in an immersion chiller, then exited onto a belt. The chiller was counter-current with 25–30 ppm chlorine added to intake water. This ensured 3–5 ppm chlorine in the exit overflow water. USDA regulations require ≈ 2L of chilled water added to the chiller for each bird.

Sampling plan

Specimens were collected by a random time sampling plan. The daily schedule of operations was ≈ 8 hr. The postchill site was sampled 1 hr later than preevisceration and prechill sites, because the required time for product to enter this site lagged about an hr after that for the first two sites. Days were divided into four 2-hr blocks. Each 2-hr block was divided into four 30-min segments. A 30-min segment was chosen randomly for specimen collection during each 2-hr block. Three or four specimens were chosen at sites 1 and 2 during the selected 30-min segment. Specimens were taken alternately from each line at sites where there were two distinct lines (preevisceration and prechill). Twelve or

Table 1—Means of $\log_{10}$ aerobic plate counts								
		Slaughter line speed (Birds per minute)[a]						
Site	n	70	80	90	100A[c]	100B[c]	100C[c]	Linear trend[b]
1	50[d]	3.79	3.62	3.63	3.79	3.55[a]	3.62	.00
2	50	3.02	3.11	3.21	3.40*	3.07	3.21	.13*
3	200[e]	2.61	2.62	2.73	2.82*	2.60	2.27*	.07

[a] Standard errors for calculation of planned comparisons of paired means were estimated by pooling across day and within week with 18 degrees of freedom.
[b] Linear trend (regression coefficient) shows expected change in mean counts for every 10 bird/min increase in line speed — calculated using first 4 wk of data with 2 degrees of freedom. Linear trends is significantly different (*) from zero at P<.01.
[c] 100A, 100B, 100C were collected at different times
  100A: Aug. 6-9, 1990; 100B: Feb. 18-21, 1991; 100C: May 13-16, 1991
[d] Sample size (n) at line speed 70 = 49. One sample broken in transit.
[e] Sample size (n) at line speed 90 = 195; at 100C line speed = 198. Some samples were broken in transit for 90 and 100C trials.

Table 3—Means of $\log_{10}$ E coli counts								
		Slaughter line speed (Birds per minute)[a]						
Site	n	70	80	90	100A[c]	100B[c]	100C[c]	Linear trend[b]
1	50[d]	2.33	2.29	2.34	2.42	2.47	2.42	0.02
2	50	1.66	1.99	1.95	1.89	2.05	1.93	0.07
3	200[e]	1.12	1.22	1.36	1.46	1.36	0.69*	0.09*

[a] Standard errors for ca culation of planned comparisons of paired means were estimated by pooling across day and within week with 18 degrees of freedom.
[b] Linear trend (regression coefficient) shows expected change in mean counts for every 10 birds/min increase in line speed — calculated using first 4 wk of data with 2 degrees of freedom. Linear trend is significantly different (*) from zero at P<.01.
[c] 100A, 100B, 100C were collected at different times 100A: Aug. 6-9, 1990; 100B: Feb. 18-21, 1991; 100C: May 13-16, 1991
[d] Sample size (n) at line speed 70 = 49. One sample was broken in transit.
[e] Sample size (n) at line speed 90 = 195; at 100C line speed = 198. Some samples were broken in transit for 90 and 100C trials.

Table 2—Means of $\log_{10}$ Enterobacteriaceae counts								
		Slaughter line speed (Birds per minute)[a]						
Site	n	70	80	90	100A[c]	100B[c]	100C[c]	Linear trend[b]
1	50[d]	2.94	2.83	2.87	3.16	2.86	2.92	0.06
2	50	2.14	2.43	2.41	2.55	2.45	2.38	0.12
3	200[d]	1.69	1.69	1.84	2.01*	1.84	1.25*	0.11

[a] Standard errors for calculation of planned comparisons of paired means were estimated by pooling across day and within week with 18 degrees of freedom.
[b] Linear trend (regression coefficient) shows expected change in mean counts for every 10 birds/min increase in line speed — calculated using first 4 wk of data with 2 degrees of freedom. Linear trend is significantly different (*) from zero at P<.01.
[c] 100A, 100B, 100C were collected at different times 100A: Aug. 6-9, 1990; 100B: Feb. 18-21, 1991; 100C: May 13-16, 1991
[d] Sample size (n) at line speed 70 = 49. One sample broken in transit.
[e] Sample size (n) at line speed 90 = 195; at 100C line speed = 198. Some samples broken in transit for 90 and 100C trials.

13 specimens were selected alternately from each side of the system at postchill during each selected 30-min segment. Sample numbers were determined by taking an equal number of required samples for each time segment, each day to reach the required number of samples. To generate significant data USDA statisticians recommended 50 carcass rinse samples at each line speed for sites 1 and 2. Carcasses at site 3 were ready-to-cook, therefore, their microbiological quality was of the highest importance. Samples (200) were collected at postchill for each line-speed trial to increase sensitivity, especially for salmonellae.

Carcass rinse specimens (50) were collected at preevisceration and 50 at prechill. Two hundred specimens were collected at postchill. A total of 300 carcass rinse specimens were collected, over 4 days for each line speed. Total specimens were 300 at 70 BPM, 300 at 80 BPM, 300 at 90 BPM, and 300 rinses for each replication of 100 BPM. Specimens for 70 BPM were collected June 14, 15, 18, and 19, 1990. Specimens for 80 BPM were collected June 27, 28, and July 9, 10, 1990. Specimens for 90 BPM were collected July 11, 12, 13, and 16, 1990. Three replications of specimens were collected for 100 BPM, (A) August 6-9, 1990, (B) February 18-21, 1991, and (C) May 13-16, 1991. Thus a total of 1,800 carcass rinse specimens were collected and analyzed for this study.

Sample preparation

A whole carcass rinse technique was used on each carcass selected at the appropriate time and site (Surkiewicz et al., 1969). Each carcass was grasped by an operator (wearing a new pair of plastic gloves) and excess water was allowed to drip from the carcass. The carcass was then placed in a new plastic bag, 200 mL of sterile phosphate buffer solution was poured on and/or into the cavity of the carcass, and the carcass was shaken by hand for 1 min. The rinse was drained aseptically into a sterile 250-mL plastic bottle. Rinse specimens were labeled with an identification number, frozen immediately, and shipped air express under dry ice in insulated shipping containers to the FSIS Eastern Laboratory in Athens, GA. The laboratory was not advised of the site or time of specimen collections.

Laboratory procedures

Aerobic plate counts. Decimal dilutions (10 mL of rinsings plus 90 mL of phosphate buffer solution) were prepared. Duplicate pour plates (Difco plate count agar, Difco Laboratories, Detroit, MI) from each di-

lution were prepared, incubated at 35°C for 48 hr, and then counted. Colony-forming units were calculated and reported as counts/mL of rinse solution (*Microbiology Laboratory Guide Book*, 1974).

Enterobacteriaceae count. Prepared dilutions were spread-plated on peptone Tergitol glucuronide agar plates and incubated at 35°C for 24 hr, and all colonies were counted. Counts were recorded as Enterobacteriaceae colonies/mL of rinse solution (Damare et al., 1985).

Escherichia coli **count.** The same peptone Tergitol glucuronide agar plates were examined under UV light. Flourescent colonies were enumerated and reported as *E. coli*/mL of rinse solution (Damare et al., 1985).

Salmonellae. The remaining rinse volume was added to sterile lactose broth containing 0.6% Tergitol. Each specimen was incubated at 35°C and, after 24 hr, 0.5 mL of the solution was transferred to a 10-mL tube of tetrathionate broth and incubated for 18 hr at 43°C. Brilliant green sulfa agar plates and modified lysine iron agar plates were streaked and incubated at 35°C for 24 and 48 hr, respectively. Typical colonies then were picked to lysine iron agar slants and triple sugar iron agar slants. Colonies with typical lysine iron and triple sugar iron slant reactions were subjected to further biochemical and serologic identification testing (*Microbiology Laboratory Guide Book*, 1974). Salmonellae was reported as present or absent.

Data analysis. $\log_{10}$ of bacterial plate counts were used in all statistical analyses. *Salmonella* prevalence was transformed to a 0/1 variable, with 0 indicating a negative finding, and 1 indicating a positive finding.

The possible relationship between processing line speeds measured as BPM and bacterial counts in whole bird rinses was investigated. The mean $\log_{10}$ bacterial counts of samples collected at 80, 90, and 100 BPM were compared to mean counts of samples collected at 70 BPM using a "t" test. *Salmonella* were not enumerated, consequently a percent of *Salmonella* positive rinses was used as the basis for comparison of *Salmonella* from whole bird rinses. Calculations were performed by SAS procedures (SAS Institute, Inc., 1990).

RESULTS

MEAN COUNTS for APC, Enterobacteriaceae, *E. coli*, and *Salmonella* at 80, 90, and 100 BPM were compared with mean counts at 70 BPM (Tables 1–4). For APC, Enterobacteriaceae, and *E. coli* means of counts from site 1, 2, and 3 generally maintained the same relationship to each other (Tables 1–3). Notable exceptions were the means at site 3 for line speed 100C that dropped sharply compared to means at sites 1 and 2. This may have resulted from an increase in water used in the inside-outside bird washer. A correction was made to methodology for line speed 100C. It was determined that water used in this equipment was not increased during trials 100A and 100B in proportion to previous increases in line speeds. The water flow was corrected proportionally during trial 100C to equal that per bird used during 70, 80, and 90 BPM.

There were two significant increases and one significant decrease for APC counts from the base mean of 70 BPM at the three sites (Table 1). Enterobacteriaceae counts showed one significant increase and one significant decrease (Table 2). *E. coli* counts showed no significant increase and one significant decrease (Table 3). *Salmonella* prevalence showed one significant increase and no significant decrease (Table 4).

Table 4—Percentages of rinses with Salmonella

| | | Slaughter line speed (Birds per minute)[a] | | | | | | |
Site	n	70	80	90	100A[c]	100B[c]	100C[3]	Linear trend[b]
1	50[d]	36.00	20.41	46.00	44.00	50.00	24.00	4.95
2	50	34.00	28.00	20.00	30.00	62.00*	14.00	1.79
3	200[e]	22.50	22.50	34.87	29.00	62.31	8.08	3.19

[a] Standards errors for calculation of planned comparisons of paired means were estimated by pooling across day and within week with 18 degrees of freedom.

[b] Linear trend (regression coefficient) shows expected change in mean counts for every 10 birds/min increase in line speed — calculated using first 4 wk of data with 2 degrees of freedom. Linear trend is significantly different (*) from zero at $P<.01$.

[c] 100A, 100B, 100C were collected at different times 100A: Aug. 6-9, 1990; 100B: Feb. 18-21, 1991; 100C: May 13-16, 1991

[d] Sample size (n) at line speed 70 = 49. One sample was broken in transit.

[e] Sample size (n) at line speed 90 = 195; at 100C line speed = 198. Some samples were broken in transit for 90 and 100C trials.

Mean bacterial counts at sites 1, 2, and 3 within each line speed repetition were significantly different for APC, Enterobacteriaceae, and *E. coli* with one exception. The difference between mean *E. coli* counts at site 1 and site 2 (2.29 and 1.99) at line-speed 80 were not different. The *Salmonella* prevalences were not significantly different between sites within any line speed, except at line speed 100C in which the prevalence at site 3 (8%) was significantly less than at site 1 or 2 (24.0% and 14.0%).

DISCUSSION

THERE WERE NO SIGNIFICANT differences in bacterial contamination at site 1 for any line speed compared to 70 BPM. This absence of change indicates the scalding and picking processes were unaffected by line speed, as long as proportionate changes in scalder water were made.

At site 2, a positive linear trend occurred for linespeed and APC. No trend was demonstrated for Enterobacteriaceae, *E. coli*, or *Salmonella*. The trend for APC could be attributed primarily to the significant increase during trial 100A (Table 1).

At site 3, a positive linear trend occurred between line speed, and *E. coli*. A similar correlation was not demonstrated for APC or Enterobacteriaceae, or *Salmonella*. Reductions in bacterial counts from site 2 to site 3 were significant and differences in means of bacterial counts between sites 2 and 3 for all line speed trials were similar in magnitude. This is indicates the chilling process was unaffected by changes in line speed as long as proportionate changes in chlorinated chiller water were made concurrently. Regulations require ≈ 2L of chilled water per carcass.

Carcasses were cleaner at each successive site in the process at all line speeds for all three bacterial counts (Tables 1-3). All

reductions were significant except from site 1 to site 2 for *E. coli* for linespeed trial 80. These results were consistent with previous reports (James, 1992a, b; Lillard, 1990).

None of the trials demonstrated a significant change in the proportion of carcasses with *Salmonella* at successive sites for any line speed.

Our results indicate that changes in processing line speed did not result in changes of bacterial contamination of ready-to-cook broilers. Mean bacterial counts on broiler carcasses did not increase significantly with increases in slaughter line speeds. No changes were made in the slaughter process except for amounts of water used in scalders, inside/outside bird washers, and chiller proportionate to changes in line speed. Significant reductions compared to 70 BPM were found during trial 100C at site 3 for APC, Enterobacteriaceae, and *Salmonella*.

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Foaming Properties of Selected Plant and Animal Proteins

BOLNEDI VANI and J.F. ZAYAS

ABSTRACT

The foaming properties of proteins are important in predicting their functionality in aerated foods. In model aqueous systems, foam expansion (FE) and foam stability (FS) of commercial plant proteins, wheat germ protein flour (WGPF), corn germ protein flour (CGPF), and soy flour (SF), were compared with those of nonfat dried milk (NFDM) and egg white powder (EWP) at 1, 2, 4, 6, and 8% using one- and two-way analyses of variance. The effects of pH 4, 5, 6, 7, and 8 on FE and FS of WGPF were also measured. The highest overall FE and FS were obtained for EWP. Among plant proteins, FE and FS were maximum for CGPF and SF, respectively. FS was lowest for NFDM. Except for SF, FE and FS increased with increasing protein concentration. The FE and FS of WGPF were highest at pH 8, lowest at pH 7, and intermediate at pH 4—6.

Key Words: wheat germ, foam expansion, foam stability, plant protein, corn germ

INTRODUCTION

PROTEIN SOURCES that can replace egg whites are in great demand because of the cost of egg products (To et al., 1985). Novel proteins with flexible functional properties facilitate the fabrication of protein-enriched products for consumers and for a wide range of markets (Kinsella, 1976). Proteins of plant and animal origin differ in their ability to stabilize foams, reflecting differences in composition, conformation, molecular flexibility, and physicochemical properties (Elizalde et al., 1991). Amino acid sequence and disposition; molecular size, shape, conformation, and flexibility; surface polarity; charge; and hydrophobicity all influence foaming behavior of food proteins. These, in turn, are affected by processing history and the physical and chemical conditions under which the protein is used. Understanding the molecular properties accounting for optimum foaming characteristics enables food scientists to select appropriate proteins and to predict surfactant properties of food proteins (Kinsella, 1981). Protein foams are important in many processes in the beverage and food industries, and this has stimulated interest in their formation and stability. Foams are used to improve texture, consistency, and appearance of food. Foams in food systems are found commonly in baked, confectionery, and other goods (Kitabatake and Doi, 1982).

The objective of our study was to investigate the foaming properties of selected plant and animal protein preparations in a model system. Specific objectives were to: (1) compare foaming properties of plant proteins—wheat germ protein flour (WGPF), corn germ protein flour (CGPF), and soy flour (SF) with those of animal proteins—nonfat dried milk (NFDM) and egg white powder (EWP); (2) study the effect of concentration of protein preparations (1, 2, 4, 6, and 8%) on foam expansion (FE) (30 sec) and foam stability (FS) (30 min, 1, 1.5, and 2 hr); and (3) study the effects of pH (4, 5, 6, 7, and 8) on foaming properties of WGPF at fixed (1%) concentration.

MATERIALS & METHODS

Raw materials

Commercial samples of defatted WGPF (Vitamins Incorporation, Chicago, IL); CGPF (Dry Corn Milling Industry); SF (Central Soya Com-

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pany, Fort Wayne, IN); NFDM (Carnation, Nestle Food Co., Glendale, CA); and EWP (M lton G. Waldbaum, NE) were used.

Proximate analysis, specific gravity and color

AOAC (1984) methods were used to determine protein (2.057, 14.026), fat (Fosslet apparatus, Foss Food Technology, MN), moisture (14.004), and ash (14.006) of WGPF. Protein content of WGPF, CGPF, SF, NFDM, and EWP was determined by the Buchi semi-macro-Kjeldahl method (AOAC, 1984). Correction factors of 5.70 and 6.25 were respectively used for plant and animal proteins. Color was determined with a D-54 spectrophotometer using the procedure used by Gnanasambandam and Zayas (1992). Specific gravity of WGPF was obtained by dividing the mass of WGPF by the volume of distilled water contained in a Fisher pycnometer.

Foaming capacity and stability

Foaming properties of the three plant proteins, WGPF, CGPF and SF, were compared with those of animal proteins, NFDM and EWP, at 1, 2, 4, 6, and 8% levels. Foaming properties of proteins were determined by the method of Wang and Kinsella (1976). Proteins at fixed concentration (1—8%) were dispersed in 50 mL distilled water and blended in an Osterizer Galaxie blender for 30 sec. The volume of foam in mL measured at 30 sec was reported as FE. The volume of foam after incubation for 30 min, 1, 1.5, and 2 hr was expressed as FS. The FE and FS of WGPF at 1% level was determined by adjusting pH to 4, 5, 6, 7, or 8.

Statistical analysis

One- and 2-way analyses of variance were carried out using an SAS Institute, Inc. (1985) computer package. Fisher's least significant difference (LSD) was used to analyze differences (P ≤ 0.05). Tests were replicated three times.

RESULTS & DISCUSSION

Proximate composition, specific gravity, and color of WGPF

The proximate composition of defatted WGPF was determined (Table 1). WGPF is a low cost and concentrated source of proteins and minerals. Low fat and moisture contents of WGPF indicate its potential for high storage stability. The carbohydrate content of germ varies widely (19.2—53.0%) depending on amount of inclusion of endosperm and bran portions and therefore is the best index of germ purity (Grewe and LeClerc, 1943). The high carbohydrate content arises from the high starch, crude fiber and sugars. Sucrose (57.6%), raffinose (37.6%), fructose (4.8%) and traces of glucose formed the major components of the germ while endosperm was high in starch and the bran was high in cellulose and hemicellulose (Dubois et al., 1960). Specific gravity is influenced by the content of bran (cellulose and hemicellulose) and starch from endosperm

Table 1—Proximate composition, specific gravity, and color of wheat germ protein flour

Parameter	Value	Parameter	Value
Moisture, %	4.36 ± 0.05	Specific gravity	0.835
Fat, %	0.53 ± 0.11	L	74.45
Protein, %	29.8 ± 0.18	a	1.91
Ash, %	4.59 ± 0.12	b	13.96
Carbohydrates, %	60.72 ± 0.34	Saturation index	14.09
		Hue angle	82.20

Table 2—Foam expansion (mL/30 sec) of wheat germ protein flour (WGPF), corn germ protein flour (CGPF), soy flour (SF), nonfat dry milk (NFDM) and egg white powder (EWP) in aqueous systems

Conc (%)	WGPF	CGPF	SF	NFDM	EWP
1	15.0^{jk}	14.1^k	18.1^i	25.0^f	6.0^l
2	14.3^k	17.3^{ij}	19.6^{hi}	26.6^f	30.0^e
4	19.5^{hi}	24.0^{fg}	14.6^{jk}	30.6^{de}	33.3^d
6	25.0^f	25.5^f	14.0^k	32.3^{de}	50.3^b
8	21.3^{gh}	26.3^f	13.3^k	37.0^c	75.0^a

$^{a-l}$ Means with different superscript significantly different (p < 0.05).

Table 3—Foam stability (mL/30 min) of wheat germ protein flour (WGPF), corn germ protein flour (CGPF), soy flour (SF), nonfat dry milk (NFDM) and egg white powder (EWP) in aqueous systems

Conc (%)	WGPF	CGPF	SF	NFDM	EWP
1	5.6^{kl}	5.8^k	15.0^{gh}	2.8^{lm}	4.8^{kl}
2	5.8^k	9.0^j	17.0^{fg}	0.5^m	25.6^d
4	9.0^j	16.3^g	12.5^{hi}	19.3^{ef}	17.6^{efg}
6	12.6^{hi}	20.1^e	12.6^{hi}	24.6^d	37.6^b
8	15.1^{gh}	20.3^e	11.6^{ij}	28.6^c	44.1^a

$^{a-m}$ Means with different superscript significantly different (p < 0.05).

Table 4—Means for foam expansion, FE and foam stability, FS (mL) of wheat germ protein flour (WGPF), corn germ protein flour (CGPF), soy flour (SF), nonfat dry milk (NFDM) and egg white powder (EWP) in aqueous systems

Source	FE, 30 sec	FS, 0.5 hr	FS, 1 hr	FS, 1.5 hr	FS, 2 hr
WGPF	19.0^d	9.6^d	7.3^d	6.5^d	5.9^d
CGPF	21.4^c	14.3^{bc}	11.7^c	10.4^c	9.7^c
SF	15.9^e	13.7^c	12.9^b	12.5^b	11.8^b
NFDM	30.3^b	15.2^b	5.8^e	5.2^e	4.9^d
EWP	38.9^a	26.0^a	23.3^a	21.0^a	18.8^a

$^{a-e}$ Values are overall means for proteins at 1, 2, 4, 6, and 8% levels. Means in the same column with different superscript significantly different (p < 0.05).

admixed with wheat germ protein. The color of WGPF is important when evaluating its potential applications in foods. The high L value (74.4) and pale yellow color (hue angle = 82.2, saturation index = 14.1) of WGPF indicate that its use in food should not pose notable color problems.

Protein content

The protein contents of defatted WGPF, CGPF, SF, NFDM, and EWP were 29.8% ± 0.1, 18.9% ± 0.3, 48.7% ± 0.3, 32.2% ± 0.1, and 80.0% ± 0.5, respectively. Of these, EWP had the highest protein content. Among plant proteins, SF had the highest protein content and WGPF was second.

Effect of protein concentration on foam expansion

The effects of source of proteins and concentration on FE were compared (Table 2). FE increased with increasing concentrations of CGPF, NFDM, and EWP, whereas it peaked at the 6% level for WGPF and decreased at ≥ 4% level for SF. Overall, the highest FE was obtained for EWP at 8% and the lowest at 1%. In comparison to other protein sources, increasing concentrations of EWP caused a greater increase in FE. Catalytic and functional properties of polymers are determined not only by their composition and structure but also by their dynamic properties (Frauenfelder and Ormos, 1988). Proteins of different origins varied greatly in foaming properties, reflecting differences in amino acid sequence and disposition; molecular size, shape, conformation and flexibility; surface polarity; charge; and hydrophobicity (Table 2).

A notable part of the effect of concentration could be related to changes in viscosity of the continuous phase. The increase in viscosity with increasing concentrations of proteins was visually obvious. Sometimes high viscosity can completely prevent incorporation of air into liquid (Peter and Bell, 1930). FE had
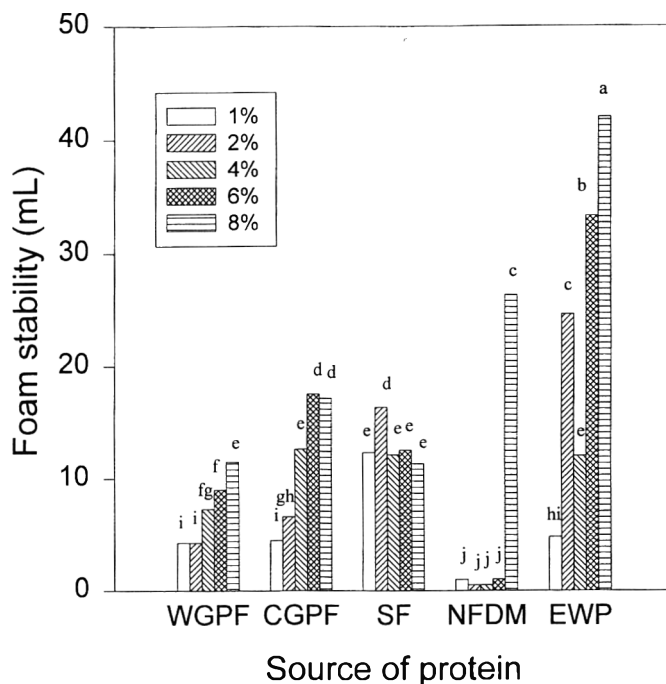

Fig. 1—Effect of protein concentration on foam stability of proteins after 1 hr incubation.

been reported to increase with increasing concentration up to a limit, and then decrease (Richert, 1979; Cherry and McWatters, 1981). High viscosity may have been responsible for the decline in overrun of WGPF and SF at higher protein concentrations. The FE of SF increased with concentration up to a limit of 2%, above which it decreased. At low concentrations and low surface pressures, ample area is available for each molecule to extend and spread on the interface (Graham and Phillips, 1979a). As surface concentration and pressure are increased, folding and looping of protein become pronounced. Adsorption is rapid at high protein concentration or in stirred systems (Kinsella, 1981). Formation of multilayers above a given protein concentration, can be regarded as gelled protein layers whose rheological properties depend upon the degree of crosslinking (disulfide linkages) and intermolecular interactions (Kinsella, 1981). There appeared to be optimum concentrations of 6% and 2%, respectively for maximum FE of WGPF and SF. But for the other proteins (CGPF, NFDM, and EWP) the optimum concentration was probably not reached at the tested levels (Table 2).

Effect of protein concentration on foam stability

The FS of WGPF, CGPF, NFDM and EWP after 30 min incubation was improved at higher levels (1—8%) (Table 3) SF had the highest FS at the 2% level, and thereafter its stability decreased. Thicker and more rigid interfacial films at higher protein concentrations of WGPF, CGPF, NFDM, and EWP may have improved foam stability by reducing the serum flow within the lamella structure (Kinsella, 1981; Phillips, 1981). Drainage may be influenced directly by bulk density and viscosity of lamellar fluid. Drainage of lamellar fluid with incubation of foam for 30 min, 1, 1.5, and 2 hr was lowest at 8% concentration for all proteins, except SF. Surface concentration might have affected the surface pressure (Graham and Phillips, 1979b), the magnitude of which might vary considerably with different proteins because of different molecular compositions and properties. According to Richert (1979) and Halling (1981) increasing protein concentration resulted in decreased drainage.

Proteins varied in foam stabilization properties (Table 4, Fig. 1, 2 and 3). The effect of protein concentration on serum drainage was dependent on type of protein used to stabilize the foam. WGPF, CGPF, and EWP showed high FS (1, 1.5, and 2 hr
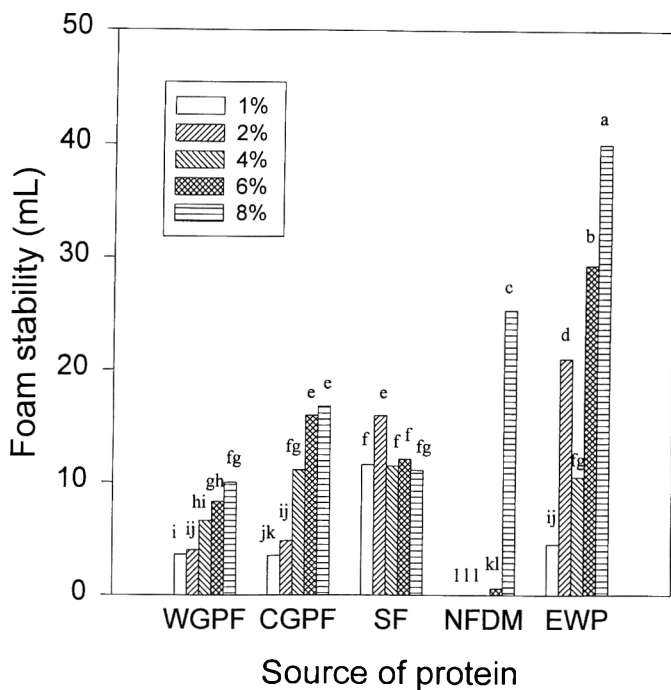
Fig. 2—Effect of protein concentration on foam stability of proteins after 1.5 hr incubation.
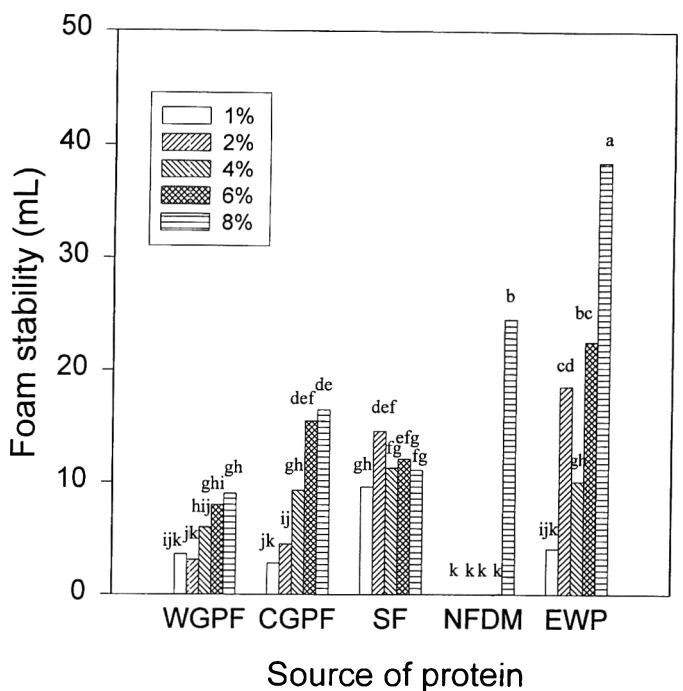

Fig. 3—Effect of protein concentration on foam stability of proteins after 2 hr incubation.

incubations) at higher levels, but a slight decrease occurred in FS of SF at higher levels (4—8%) (Fig. 1, 2, and 3). FS was dependent on the ability of the protein to form a strong, flexible, cohesive film to reduce gas permeability and inhibit bubble coalescence. Proteins with good foamability do not necessarily give good foam stability and vice versa. Foam prepared with NFDM retained its stability only at the 8% level (Fig. 1, 2, and 3) with 1, 1.5, and 2 hr incubations. Two-way ANOVA (Table 4) showed that FE and FS (up to 2 hr) were highest for EWP. Among plant proteins, CGPF had the highest FE and SF had the highest FS (up to 2 hr). Of all proteins, the FS of NFDM after 1 hr was the least. Studies of foaming behavior of proteins are complicated by interactions of mixtures rather than individual proteins and by molecular changes resulting from processing treatments. In homogeneous protein systems, the predominant attractive forces between proteins are short-range hydrogen bonds, hydrophobic, electrostatic, and Van der Waals interactions (Kinsella, 1984; Poole et al., 1984). However, in heterogeneous systems, factors such as isoelectric point and molecular charges arising from commercial processing of proteins and other components (such as fats) can cause variations.

Effect of pH on foam expansion and foam stability

Chemical modification may improve FS by changing the net charge on a protein (Whitehead, 1988; Phillips et al., 1989). An attempt was made to improve the FE and FS of WGPF by altering pH. The pH of the unadjusted WGPF at the 1% level was 6.5 ± 0.1. The highest FE and FS were obtained at pH 8, and the lowest was at pH 7 (Table 5). The pH of the dispersing medium markedly affected foaming of WGPF by its direct effect on net charge and conformation of proteins. Several types of chemical and physical forces together can influence size, shape, and solubilities of proteins. Physical forces (electrostatic attraction and hydrogen or hydrophobic bonding) but not covalent bonds can be broken by specific solvents, changing pH, raising temperature or altering salt content. Both pH and temperature caused changes in conformation and structure of whey proteins (Mohan Reddy et al., 1988). The pH probably influenced active SH groups (Lee et al., 1990) and protein solubility of WGPF (Vani and Zayas, 1995). The viscosity of WGPF may have increased at slightly alkaline pH 8, as was observed with increase

Table 5—Foam expansion, FE and foam stability, FS (mL) of wheat germ protein flour (1% concentration) at various pHs

pH	FE, 30 sec	FS, 0.5 hr	FS, 1 hr	FS, 1.5 hr	FS, 2 hr
4	9.5[c]	5.5[b]	4.6[bc]	4.5[bc]	4.0[bc]
5	10.8[b]	6.5[b]	6.0[b]	5.5[b]	4.8[b]
6	10.3[bc]	5.6[b]	4.3[bc]	4.0[bc]	4.0[bc]
7	8.0[d]	4.3[b]	3.6[c]	3.1[c]	3.1[c]
8	17.3[a]	11.8[a]	9.5[a]	8.3[a]	7.3[a]

[a-d] Means in the same column with different superscript significantly different ($p < 0.05$).

in temperature (6, 18, 30, and 42°C) and pH (5—10) of egg white solutions (Pitsilis et al., 1975). Li-Chan et al. (1984) reported that hydrophobicity was influenced by a small change of pH. Mita et al. (1977) showed that surface tensions of wheat proteins were lowest in the range of pIs (pH 6.5—7.5). The FE and FS at pH 4 was slightly higher than at pH 7, but lower than those at pH 5 or 6. The protein solubility of WGPF was maximum at pH 8 and minimum at pH 4 (Vani and Zayas, 1995). Similar results were reported by others. Increases in protein solubility of high linseed mucilage concentrates on the acidic side of the pI were related to interactions between proteins and acidic pentosans (Morr and Foegeding, 1990). Studies of the surface rheology of protein films at the air-water interface indicated a maximum rigidity near the pI (MacRitchie, 1980). However, Meste et al. (1990) observed that near the isoelectric point proteins had higher flexibility and improved surface properties. Thus, both protein solubility and hydrophobicity can affect foaming properties. However, mixed systems such as WGPF are much more complex than pure protein systems because of the extra intermolecular interactions and competitive adsorption between components. Heat treatment may affect foaming via effects on protein structure and viscosity of the aqueous phase. The rate of foaming after heating may be higher for flexible randomly structured proteins than for those with tightly held tertiary structures. Studying the effect of heat denaturation on the foaming properties of WGPF has potential to improve them.

CONCLUSION

HIGHEST FOAM EXPANSION and foam stability during 2 hr of incubation were found for egg white powder. Among plant pro-

teins, CGPF showed highest foam expansion and maximum FS for up to 30 min incubation and SF displayed maximum FS after 1 hr incubation. Foam stability was lowest for nonfat dried milk after 1 hr of incubation. Overall, foam expansion and foam stability increased with increases in protein concentration (1— 8%). Adjusting pH of WGPF altered its foaming properties. Foam expansion and foam stability (up to 2 hr) for WGPF were highest at pH 8, lowest at pH 7, and intermediate at pH 4—6.

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Ms received 12/27/94; revised 4/27/95; accepted 5/20/95.

Lipoxygenase in Sweet Corn Germ: Isolation and Physicochemical Properties

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—————————— ABSTRACT ——————————

Off-flavor and off-aroma development, which may be catalyzed by lipoxygenase (LPO), are common in frozen stored sweet corn. Lipoxygenase activity in the germ fraction of sweet corn (Zea mays L. cv. Jubilee) was determined and compared with that in the degermed fraction. Lipoxygenase activity/g germ was about three times greater than that of the degermed fraction. Optimized procedures for isolation of lipoxygenase from the germ fraction were developed. Lipoxygenase was isolated by preparation as an acetone powder, extraction with 0.2M Tris-HCl, pH 8.0 (4°C), fractionation with 40–60% saturated ammonium sulfate and dialysis. Optimum pH was 6–7 and temperature 50°C for activity of partially purified lipoxygenase. The enzyme appeared stable at pH 5–8 and ≈90% of original activity was inactivated after heating in pH 7 buffer at 70°C for 3 min.

Key Words: lipoxygenase, sweet corn, germ, isolation, extraction

INTRODUCTION

LIPOXYGENASE (Linoleate: oxygen oxidoreductase, EC 1.13.11.12; previously known as lipoxidase), LPO, is an important endogenous enzyme which may be the cause of off-flavor development in unblanched or underblanched frozen vegetables including sweet corn (Wagenknecht, 1959; Lee, 1981; Williams et al., 1986; Ganthavorn and Powers, 1989; Velasco et al., 1989; Sheu and Chen, 1991). Off-flavor and off-aroma development are considered primary causes of rapid quality deterioration in harvested sweet corn (Smittle et al., 1972). Because lipoxygenase is important in quality deterioration, it may be a more appropriate indicator than peroxidase in determination of effective heat treatments (Williams et al., 1986).

According to Wagenknecht (1959) and Lee (1981), lipoxygenase induced off-flavors in germ fractions of unblanched sweet corn. Previous work on corn germ lipoxygenase has focused on mature field corn seeds (Gardner, 1970; Egmond et al., 1972; Veldink et al., 1972; Gardner and Weisleder, 1970; Gardner et al., 1975). Some sweet corn lipoxygenase studies have not emphasized the germ fraction (Wagenknecht, 1959; Lee, 1981; Velasco et al., 1989); nor have previous investigators evaluated lipoxygenase from sweet corn (Zea mays L. cv. Jubilee) germ at the stage of maturity appropriate for the freezing industry.

Our objectives were: to determine lipoxygenase activity in germ and degermed fractions of sweet corn; to develop procedures for rapid isolation of corn germ; to optimize procedures and conditions for extraction and isolation of lipoxygenase and investigate its physicochemical properties.

MATERIALS & METHODS

Materials

Sweet corn (Zea mays L. cv. Jubilee) was harvested at appropriate maturity and obtained from a commercial processor in Woodburn, OR. The corn was immediately transported on ice to the Pilot Plant of the Dept. of Food Science & Technology, Oregon State Univ., Corvallis,

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OR. Fresh sweet corn was temporarily stored at 4°C prior to germ separation, which was carried out over a period of 3 days. A portion of sweet corn was frozen immediately in liquid nitrogen and stored at −80°C until determination of lipoxygenase activity in the germ and degermed fractions.

Linoleic acid, Tween-20, Triton X-100, ammonium sulfate, and bovine serum albumin were purchased from Sigma Chemical Co., St. Louis, MO. All other chemicals were reagent grade. Deionized distilled water was used in all experiments.

Lipoxygenase activity of sweet corn germ and degermed fractions

Sweet corn ears were frozen in liquid nitrogen and intact kernels were removed from cobs. While frozen, corn kernels were separated into germ and degermed fractions and weighed. Each fraction was blended in liquid nitrogen. The liquid nitrogen powder of each fraction was homogenized in 0.2M Tris-HCl, pH 8.0 (4°C) at a ratio of 1:10 (w/v) using a Tissumizer (Tekmar Co., Cincinnati, OH), and a powerstat setting of 50 for 3 min. The extract was centrifuged at 17,000 × g (4°C) for 1 hr. The lipoxygenase activity in each supernatant was determined spectrophotometrically by monitoring the formation of conjugated dienes at 234 nm.

Lipoxygenase activity and protein assays

Lipoxygenase activity was determined spectrophotometrically at 234 nm by a modification of the procedure described by Chen and Whitaker (1986). The substrate solution was prepared by mixing 157.2 μL of pure linoleic acid, 157.2 μL of Tween-20 and 10 mL of deionized distilled water. The solution was clarified by adding 1 mL of 1.0N sodium hydroxide and diluting to 200 mL with 0.2M sodium phosphate buffer, pH 7.0; giving a 2.5 mM final concentration of linoleic acid. The substrate solution was flushed with oxygen gas 2 min or longer to give an initial absorbance at 234 nm of 0.3–0.4, and allowed to equilibrate in a water bath at 25°C before activity assay. The total reaction volume was 3 mL, which contained 2.7 mL of substrate solution and 0.3 mL of enzyme solution. The initial rate of conjugated diene formation was read over the linear change in absorbance at 234 nm.

One unit of enzyme activity was defined as an increase in absorbance of 0.001 at 234 nm/min under assay conditions. The extinction coefficient (ε_m) for the conjugated diene of linoleic acid was 23,000 M^{-1} cm^{-1} (Gibian and Vanderberg, 1987). A double beam spectrophotometer (Shimadzu, UV-160, Shimadzu Corporation, Kyoto, Japan) and 1 cm path length cuvette were used.

Protein was determined by the bicinchoninic acid (BCA) method (Smith et al., 1985) using crystalline bovine serum albumin as a standard.

Rapid isolation of sweet corn germ

Rapid isolation of the germ fraction from sweet corn on the cob was achieved using a modification of the method of Fong and Smith (1985). The precooled fresh sweet corn was husked, inspected, and cut using a TUC (The United Company, Westminster, MD) corn cutter to remove the top portion of the kernels (above the germ tissue). The cutter was carefully adjusted to cut kernels again at the base where they attach to the cob. The preccoled, cut corn kernels were gently crushed with a rolling pin to release the intact germ and then sieved. Three stainless steel sieves (U.S. standard sieve series, the W.S. Tyler Co., Cleveland, OH) with mesh numbers 1/4 (6.3 mm opening), 8 (2.36 mm), and 20 (0.83 mm) were stacked with the largest pore size on top. The crushed kernels were spread on top of the sieve stack and shaken in cold 50 mM sodium phosphate buffer, pH 7. The enriched fraction of whole germ was trapped on sieve number 8. The crushed kernels containing germ

that remained on sieve number 1/4 were crushed again with a rolling pin and sieved in cold buffer as before. The enriched fractions of germ were combined and carefully spread on a screen with opening 3.17 mm, and gently sprayed with cold buffer to allow the germ to pass through the sieve.

The enriched germ fraction was further isolated by suspending it in ≈35% (w/v) sucrose in a 50 mM sodium phosphate buffer pH 7, where most of the germ and some other tissue debris floated. All floating tissues were collected and centrifuged in ≈30% (w/v) sucrose in 50 mM sodium phosphate buffer pH 7 at 2,000 × g (4°C) for 5 min to separate the germ from debris. The isolated germ fraction was inspected and sorted from the debris of non-embryonic tissues before freezing in liquid nitrogen. Frozen isolated germ were stored at −80°C.

Optimization of lipoxygenase isolation

Preparation of acetone powder. Frozen corn germ were blended in liquid nitrogen using a stainless steel Waring Blendor. The liquid nitrogen powder was then homogenized in cold acetone (−23°C) in a ratio of 1:20 (w/v) using a Tissumizer (powerstat setting at 100) for 2 min in a cold room (4°C). The slurry was filtered through a Buchner funnel with Whatman filter paper #1. The residue was rehomogenized with 10 volumes of cold acetone for 1 min and filtered; and this step was repeated. The final residue was washed with 5 volumes of cold acetone, vacuum dried at room temperature, ground and stored at −23.3°C until used.

Optimization of extraction. The following optimized procedure for extraction of lipoxygenase from sweet corn germ acetone powder was developed: acetone powder (2g) was homogenized with 0.2M Tris-HCl, pH 8.0 (4°C) in a ratio of 1:10 (w/v) for 3 min using a Tissumizer (powerstat setting 50). The enzyme extract was centrifuged at 17,000 × g for 1 hr (4°C).

To determine optimum conditions for extraction, various factors affecting extraction efficiency of lipoxygenase from acetone powder were investigated (Table 1). Experiments were performed as described, except conditions of extraction buffers were changed as indicated. All experiments were at 4°C with at least two replications.

Ammonium sulfate fractionation and dialysis. The enzyme extract was 40% saturated with ammonium sulfate with continuous stirring at 4°C for 1 hr. After centrifugation at 17,000 × g at 4°C for 30 min, the supernatant was 60% saturated with ammonium sulfate with continuous stirring at 4°C for 1 hr. The resulting precipitate after centrifugation was dissolved in a minimum volume of 50 mM Tris-HCl buffer, pH 8.0 (4°C) and dialyzed using a Spectra/Por membrane (molecular weight cutoff 10,000 daltons) against 2 L of the same buffer overnight at 4°C with one change of buffer. The dialyzed solution was centrifuged at 17,000 × g (4°C) for 1 hr. The supernatant of isolated lipoxygenase was stored at −23.3°C. The activity of lipoxygenase and protein content were determined at each step of isolation.

pH optimum for activity and pH stability

To determine the pH optimum the activity of isolated sweet corn germ lipoxygenase was determined spectrophotometrically in the range pH 3.0 to 9.0. The buffer systems were 0.2M citrate phosphate buffer, pH 3.0 to 5.0; 0.2M sodium phosphate buffer, pH 6.0 to 7.0; and 0.2M Tris-HCl, pH 8.0 to 9.0.

In the pH stability study, the enzyme was diluted 1:10 (v/v) with buffers from pH 3.0 to 10.0 and incubated in 13 × 100 mm capped test tubes in a water bath (25°C) for 30 min, then assayed for lipoxygenase activity. The buffer systems used for pH 3.0 to 9.0 were those for the pH activity study. The buffer for pH 10.0 was 0.2 M Tris-HCl buffer.

Temperature optimum for activity and stability

Elucidation of the optimum temperature for activity involved spectrophotometric determination of lipoxygenase activity in 0.2M sodium phosphate buffer, pH 7.0 at 10, 20, 25, 30, 35, 40, 50, 60, and 70°C.

For the temperature stability study, the enzyme solution was diluted 1:10 (v/v) with 0.2M sodium phosphate buffer, pH 7.0 Aliquots of diluted enzyme sample were placed in 13 × 100 mm capped test tubes and incubated in a water bath at 60°C and 70°C for various times up to 30 min. Each tube was removed at a specific time, immediately cooled in ice water and assayed for lipoxygenase activity.

Table 1—Factors evaluated for optimization of extraction conditions for lipoxygenase

Factors	Conditions
Buffer pH (0.1 M)	4.5 (Sodium acetate) 7.0 (Sodium phosphate) 8.0 (Tris-HCl) 9.0 (Borate)
Buffer types (0.1 M, pH 8.0)	Sodium phosphate Tris-HCl Borate
Buffer concentrations	0.05, 0.1, and 0.2 M
Surfactant	Triton X-100 (0, 0.01, 0.05, 0.1, 0.5, 1.0 and 2.0% v/v)
Metal chelator	EDTA (5 mM)
Protease inhibitor	PMSF (5 mM)
Reducing agent	DTT (5 mM)

RESULTS & DISCUSSION

Lipoxygenase activity of sweet corn germ and degermed fractions

Lipoxygenase activity/g tissue (wet weight) in the germ fraction was ≈ three times greater than the degermed fraction (data not shown). Lipoxygenase activities were 22,520 and 7,780 units/g tissue in germ and degermed fractions, respectively. Previous work on distribution of lipoxygenase in sweet corn on the cob also reported lipoxygenase activity was highest in the germ (Wagenknecht, 1959; Lee, 1981; Lee et al., 1989). In inbred yellow dent corn, the activity of lipoxygenase was generally higher in germ (embryo) than in endosperm tissues throughout kernel development (Belefant and Fong, 1991). Gardner (1970) reported that lipoxygenase was localized primarily in the seed germ tissue of mature hybrid corn. Lipoxygenase activity was found primarily in the germ tissue of cereal seeds such as wheat (Auerman et al., 1971; Von Ceumern and Hartfiel, 1984), barley (Lulai and Baker, 1976; Lulai et al., 1981), and rice (Yamamoto et al., 1980); however, the subcellular location of lipoxygenase in germ tissue has not been reported (Gardner, 1988).

Since lipoxygenase activity was higher per unit weight in the germ of sweet corn (cv. Jubilee) this may be the major site for enzymatic oxidation of polyunsaturated fatty acids. Off-flavor and off-aroma may ultimately develop in that region since polyunsaturated fatty acids have also been reported to be higher in the germ of corn kernels (Wagenknecht, 1959; Gardner and Inglett, 1971; Flora and Wiley, 1972; Pascual and Wiley, 1974; Puangnak, 1976; Weber, 1978a, 1978b).

Rapid isolation of sweet corn germ

Large quantities of sweet corn germ were required and because of seasonal availability and the tedious and time consuming nature of manual isolation, a rapid method of isolation was developed. The yield of isolated germ was about 2–3% wet weight of the cut kernels. Sucrose that was used during germ separation was tested for its effect on lipoxygenase activity and had no inhibitory effects on the enzyme (data not shown).

Optimization of lipoxygenase isolation

The preparation of an acetone powder of corn germ from liquid nitrogen powder was a means of concentrating enzyme as well as removing lipids, carotenoid pigments and other impurities. Some undesirable impurities that might act as inhibitors of lipoxygenase, (e.g., natural antioxidants), may also have been removed. This technique had also been used to solubilize membrane associated enzymes (Penefsky and Tzagoloff, 1971). Previous attempts at lipoxygenase isolation from whole kernel sweet corn (Velasco et al., 1989), mature seed corn (Poca et al., 1990), wheat germ (Nicholas et al., 1982), and most lipid containing plants such as sunflower seed (Leoni et al., 1985), English pea (Chen and Whitaker, 1986), flaxseeds (Rabinovitch-

Chable et al., 1992) also noted the benefits of making acetone powders prior to extraction.

Optimization of extraction. Factors which may affect the efficiency of lipoxygenase extraction from sweet corn germ acetone powder were investigated. Conditions for extraction of lipoxygenase were selected based on preliminary observations: (1) use of a Tissumizer for extraction was more efficient than a magnetic stirrer or Waring blender; (2) three min was sufficient for effective extraction of lipoxygenase using the Tissumizer with powerstat at 50 and acetone powder to buffer 1:10 (w/v) (4°C).

Extraction at pH 8.0, yielded both the highest total lipoxygenase activity and the highest specific activity compared with other buffers (Fig. 1). Tris-HCl buffer was most efficient at extraction of lipoxygenase and sodium phosphate. Borate buffers yielded ≈92% and 86% lipoxygenase activity relative to Tris-HCl buffer. Lipoxygenase activity was highest using Tris-HCl (pH 8.0) at 0.2 M. Buffers at 0.1M and 0.05M yielded 92% and 91% activity relative to the 0.2M buffer. Therefore, 0.2M Tris-HCl was selected for extraction of lipoxygenase from acetone powders.

The effect of surfactant (Triton X-100), a metal chelator (EDTA), a reducing agent (DTT), and a protease inhibitor (PMSF) on lipoxygenase extraction were also examined. Previous work by Grossman et al. (1969), Pinsky et al. (1971) and Boyes et al. (1992) showed that using Triton X-100 in the extraction buffer increased the yield of lipoxygenase activity. Concentrations up to 2.0% (v/v) of Triton X-100 were added to the 0.2 M Tris-HCl buffer. Triton X-100 addition decreased lipoxygenase activity in the sweet corn germ extract. Triton X-100 at 0.05%, 1.0% and 2.0% decreased lipoxygenase activity to about 64%, 32% and 5% of that in the control extract without Triton. Pinsky et al. (1971) also reported that in soybean, pea, spinach and cauliflower, addition of Triton X-100 decreased lipoxygenase activity.

Such decreased activity may be due to denaturation of the enzyme by detergent. This may result from replacement of natural lipids by detergent which could cause loss of enzymic activity. However, adding Triton X-100 increased the yield of lipoxygenase in extracts of some plants. Such increases may be due to the action of the detergent in solubilizing membrane-bound enzyme.

EDTA, a metal chelating agent, may be added to extraction buffers to bind metal ions and divalent cations that could inhibit enzymes during extraction. Lipoxygenases in cowpeas (Den and Mendoza, 1982), and chickpeas (Sanz et al., 1992) have been reported to be inhibited by metal ions. However, extraction buffers to which we added 5 mM EDTA showed about an 8% decrease in lipoxygenase activity.

EDTA has been reported to be an inhibitor of lipoxygenase in some plants e.g. broad beans (Al-Obaidy and Siddiqi, 1981) and soybeans (Chan, 1973). Lipoxygenase activity in sunflower seeds (Leoni et al., 1985) and barley (Lulai and Baker, 1976), however, was not affected by EDTA. Decreased activity as a result of EDTA addition may be due to the presence of a non-heme iron atom at the active site as in soybean lipoxygenase (Chan, 1973; Roza and Francke, 1973; Pistorius and Axelrod, 1974; Navaratnam et al., 1988; Nelson, 1988; Draheim et al., 1989). EDTA might form a complex with the iron in lipoxygenase and result in a decrease in activity.

The addition of a reducing agent (DTT) at 5 mM in the extraction buffer decreased the yield of lipoxygenase to about 54% of that in the extract without DTT. This indicated that a reducing environment was not required for stabilization of lipoxygenase activity during extraction. The reducing conditions may change or keep lipoxygenase molecules in the inactive form. Moreover, addition of 5 mM PMSF as a serine protease inhibitor did not increase extraction of lipoxygenase from sweet corn germ.

Ammonium sulfate fractionation and dialysis. The optimal ammonium sulfate saturation for fractionation of lipoxygenase was determined by increasing the saturation in 10% increments
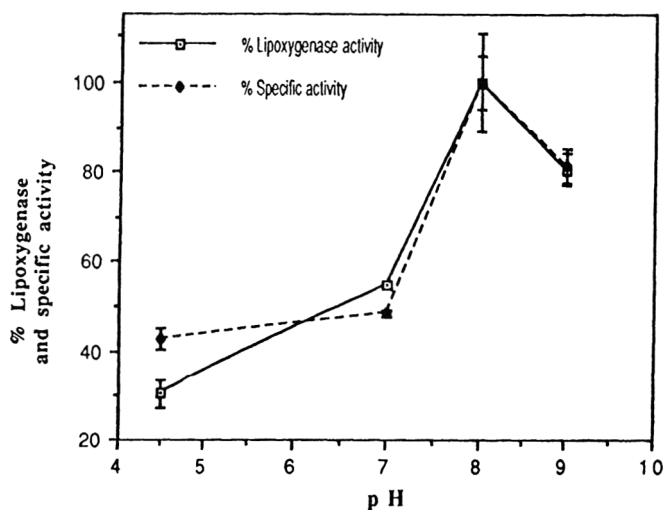

Fig. 1—**Lipoxygenase and specific activity in extracts prepared with different pH buffers.** Data are means ±SD.

from 20–100% saturation. Highest lipoxygenase activity recovery and specific activity were found between 40–60% ammonium sulfate (Table 2). This range had been used to isolate lipoxygenase from green beans (Adams and Ongley, 1989), cow peas (Den and Mendoza, 1982), and soybeans (Mitsuda et al., 1967; Steven et al., 1970).

Other ammonium sulfate saturation levels have been reported for isolation of lipoxygenase from corn, however. Belefant and Fong (1991) used 20–50% for corn embryos, Gardner and Weisleder (1970) used 42–53% for mature corn germ, Velasco et al. (1989) used 10–50% for sweet corn kernels and Poca et al. (1990) used 40–80% for mature seed of corn. The recovery was 62.1% and purification was 3-fold for isolated lipoxygenase from sweet corn germ after fractionation and dialysis (Table 2).

Optimum pH for activity and stability

The optimum pH for activity of lipoxygenase was ≈6.0–7.0 (Fig. 2). Very little activity was observed below pH 4.0 or above pH 8.0. This was similar to the pH optimum reported by Gardner (1988) for a lipoxygenase isozyme of mature maize germ which had a broad pH optimum between pH 6.0 and 7.2 and essentially no activity above pH 8.2. Belefant and Fong (1991) also reported that lipoxygenase in the embryos of inbred yellow dent corn had an optimum pH of 6.8–7.0. However, Poca et al. (1990) reported that the pH optimum for two lipoxygenase isozymes of mature maize seeds had a broad pH optimum, from pH 6.0 to 8.2 for isozyme-1 and from pH 7.0 to 9.0 for isozyme-2.

The optimum pH of lipoxygenase in the germ tissue of cereal grains has been reported to be 6.0–6.5 for wheat germ (Nicholas et al., 1982), 6.5–7.0 for rice germ (Yamamoto et al., 1980), and 6.5 for germinated barley germ (Doderer et al., 1992). Lipoxygenase activities of many other plants, e.g. broad beans (Al-Obaidy and Siddiqi, 1981), eggplant (Grossman et al., 1972), navy beans (Koch et al., 1971), soybean lipoxygenase-2 (Diel and Stan, 1978; Dreesen et al., 1982), pea isozyme-1 (Chen and Whitaker, 1986) and kiwifruit (Boyes et al., 1992) have also been found to have pH optima ranging from 6.0–7.5. However, lipoxygenase activity of soybean lipoxygenase-1 was optimum of pH 8.0–9.5 (Diel and Stan, 1978) and Reynolds and Klein (1982) found that dry English pea seeds (cv. Little Marvel) contained a small amount of type-1 lipoxygenase with optimum pH 9.0–10.0.

Sweet corn germ lipoxygenase seemed to be most stable in the range pH 5.0–8.0; however, the activity was almost entirely lost at pH 10.0 (Fig. 3). This result was similar to that reported for pea lipoxygenase isozyme-1 which was stable at pH 4.5–8.0

Table 2—Summary of partial purification of lipoxygenase from sweet corn germ

Step	Total activity (units/g AP)	Specific activity (units/mg protein)	Degree of purification	% Recovery[a]
Acetone extract	137,250	2,338	1.0	100.0 powder (AP)
40% Ammonium sulfate supernatant	105,044	2,809	1.2	76.5
40–60% Ammonium sulfate precipitate	102,375	5,543	2.4	74.6
After dialysis	95,297	7,021	3.0	62.1

[a] Relative to lipoxygenase activity in AP extract.

Fig. 3—pH stability curve for partially purified lipoxygenase from sweet corn germ. Data are means ±SD.

Fig. 2—pH activity profile for partially purified lipoxygenase from sweet corn germ. Data are means ±SD.

Fig. 4—Temperature activity profile for partially purified lipoxygenase from sweet corn germ. Data are means ±SD.

at 25°C for 30 min (Chen and Whitaker, 1986). Ganthavorn and Powers (1989) reported that asparagus lipoxygenase was also stable at pH 4.5–8.0 at 2°C for 3 days. Al-Obaidy and Siddiqi (1981) found that broad bean lipoxygenase was stable between pH 4.0 and 8.0, however, the time and temperature were not reported.

Optimum temperature for activity and stability

The optimum temperature for lipoxygenase activity was ≈50°C (Fig. 4). This was similar to the optimum reported for three major lipoxygenase isozymes of wheat germ which was ≈45°C. They had only trace activities at 65°C (Shiiba et al., 1991), and barley lipoxygenase had an optimum of 47°C (Lulai and Baker, 1976). However, this was higher than the optimum temperature found for sunflower seeds (Leoni et al., 1985) which was ≈35°C; soybeans, broad beans and cowpeas with maximum activity ≈30°C (Tappel et al., 1953; Al-Obaidy and Siddiqi, 1981; Den and Mendoza, 1982).

About 90% of the original activity of isolated lipoxygenase from sweet corn germ was inactivated (Fig. 5) after heating at 70°C for 3 min. Lipoxygenase was almost completely inactivated after 20 min at 70°C. About 50 and 90% of original lipoxygenase activity was inactivated after heating at 60°C for 3 and 20 min, respectively.

After heating 10 min, residual lipoxygenase activities were about 25% at 60°C and 5% at 70°C. Lipoxygenase in sweet corn germ seemed to be less heat stable than that in English green pea homogenate where residual activities after heating at 60°C for 10 min were about 30% (Williams et al., 1986). Poca et al.

(1990) reported that isolated lipoxygenase isozyme activities from mature maize seeds remained at about 50% after heating for 5 min at ≈45°C for isozyme-1 and at 65°C for isoenzyme-2. Results for thermal inactivation of lipoxygenase enzymes reported in other studies may not be directly comparable because of various factors, e.g. corn cultivar, technique for heating and assaying enzymes, criteria for inactivation, enzyme concentrations, amounts and types of impurities, substrates of enzymes, and reaction products (McConnell, 1956; Svensson and Eriksson, 1972; Alsoe and Alder-Nissen, 1988; Ganthavorn and Powers, 1989).

A pH 7 buffer was used for study of the heat stability of the enzyme since that is the approximate physiological pH of corn germ. However, lipoxygenase naturally present in germ may be more heat stable than that of the isolated enzyme in a buffer. A protective environment may be imparted by the kernel structure and other compounds may act as stabilizers.

CONCLUSIONS

LIPOXYGENASE CONCENTRATION was higher in the germ as compared to the degermed fraction of sweet corn, suggesting that industry should pay careful attention to the germ fraction. The germ may be an important site for enzyme-catalyzed off-flavor and off-aroma formation in unblanched sweet corn. Conditions for extraction and isolation of lipoxygenase from germ
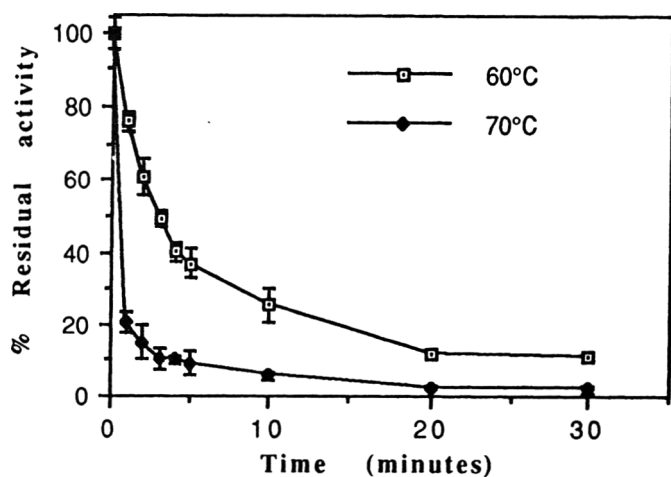
Fig. 5—Temperature stability curves for partially purified lipoxygenase from sweet corn germ. Data are means ± SD.

of sweet corn were optimized for the first time. Elucidation of physicochemical properties, such as pH and temperature optimum for activity, and pH and temperature stability provide a better understanding of lipoxygenase in germ. Continued research will lead to an understanding of the influence of lipoxygenase on sweet corn quality. Lipoxygenase may be more appropriate than peroxidase for use as an index for blanching in the frozen sweet corn industry.

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Sweet Corn Germ Enzymes Affect Odor Formation

CHOCKCHAI THEERAKULKAIT, DIANE M. BARRETT, and MINA R. McDANIEL

ABSTRACT

Descriptive sensory analysis of a homogenate of frozen stored unblanched sweet corn indicated that mean overall intensity and most descriptors describing undesirable characteristics were higher than those from blanched corn. To investigate the involvement of corn germ enzymes in off-odor formation, crude enzyme and purified lipoxygenase (LPO) and peroxidase (POD) extracts were prepared and added to homogenates of blanched corn. Addition of the LPO extract increased "painty" and "stale/oxidized" off-odor descriptors and lowered "sweet" and "corn" descriptors. Evidence suggested that sweet corn germ peroxidase is not important in off-odor formation, in which case lipoxygenase may be more appropriate as a blanching indicator.

Key Words: sweet corn, corn germ, lipoxygenase, peroxidase, off-odors

INTRODUCTION

SWEET CORN OFF-FLAVOR and off-odor formation, which occur after harvesting and during frozen storage, result in quality deterioration (Smittle et al., 1972; Wagenknecht, 1959; Lee, 1981; Velasco et al., 1989). Off-odor formation in frozen stored raw or underblanched vegetables, including sweet corn, is hypothesized to be the result of enzymatic action (Joslyn, 1949; Wagenknecht, 1959; Lee, 1981; Williams et al., 1986; Ganthavorn and Powers, 1989; Velasco et al., 1989; Sheu and Chen, 1991).

Lipoxygenase (LPO) has most often been suggested as the cause of off-flavor development (Wagenknecht, 1959; Lee, 1981; Velasco et al., 1989), and its activity is particularly high in the fraction containing the germ (Wagenknecht, 1959; Lee, 1981). In contrast, there is no evidence that peroxidase (POD), long used as a blanching index for sweet corn and other vegetables prior to freezing, is directly associated with off-flavor or other quality deterioration (Morris, 1958; Burnette, 1977; Williams, et al., 1986; Lim et al., 1989; Sheu and Chen, 1991).

Velasco et al. (1989) separated POD, LPO and catalase from sweet corn kernels and evaluated catalysis of off-odor formation by those enzymes. They could not clarify which enzyme(s) was responsible. The involvement of LPO and other enzymes isolated specifically from sweet corn germ tissue has never been reported.

Our objectives were to investigate the effects of sweet corn germ enzymes, in particular LPO, on off-odor formation using descriptive sensory analysis. The odor profiles of homogenates prepared from blanched and unblanched frozen stored corn were studied and compared to those of homogenates to which enzyme extracts had been added.

MATERIALS & METHODS

Materials

Freshly harvested sweet corn (*Zea mays* L. var. Jubilee) was obtained from the National Frozen Food Co. (Albany, OR) and was immediately transported to the pilot plant of the Dept. of Food Science and Technology, Oregon State Univ. (Corvallis, OR). The fresh sweet corn was dehusked by hand, and randomly separated and processed into four lots:

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(1) unblanched intact kernels: corn was frozen immediately in liquid nitrogen and intact kernels were removed from the cob by hand and stored at −35°C; (2) blanched intact kernels: corn on the cob was water blanched at 98°C for 30 min to ensure inactivation of enzymes, cooled in water and frozen immediately in liquid nitrogen. Intact kernels were then removed from the cob by hand and stored at −35°C until utilized; (3) unblanched corn on the cob frozen immediately in liquid nitrogen and stored at −23.3°C for up to 1.75 years for investigation of off-odor; and (4) remainder of the fresh corn temporarily stored at 4°C prior to germ separation, which was carried out over a period of 3 days.

Linoleic acid, ammonium sulfate, Tween-20 (polyoxoethylene-sorbitan monolaureate), and gel filtration media (Sephacryl S-300 HR) were purchased from Sigma Chemical Co. (St. Louis, MO). All other chemicals were reagent grade. Deionized distilled water was used in all purification experiments. A prepacked Fast Protein Liquid Chromatography (FPLC) column of Mono Q HR 5/5 (5 × 0.5 cm i.d.), and a prepacked disposable PD-10 (Sephadex G-25 M) column were obtained from Pharmacia-LKB (Uppsala, Sweden). The FPLC system (Pharmacia) was previously described by Theerakulkait and Barrett (1995a).

Reference standards for odor descriptors were linseed oil (Grumbacher, Artists Oil Medium by M. Grumbacher, Inc., New York, NY), corn tortilla mix (Quaker Masa Harina De Maiz, manufactured by the Quaker Oats Co., Chicago, IL), canned whole kernel corn (Golden sweet, family style, Del Monte brand, Del Monte Co., San Francisco, CA), dried straw and hay (Dept. of Animal Science, Oregon State University, Corvallis, OR), and fresh sweet corn and cabbage (purchased at local markets, Corvallis, OR). Reference standards for odor intensity were safflower oil (Saffola Quality Foods Inc., Los Angeles, CA), orange drink (Hi-C, Coca Cola Foods, Houston, TX), grape juice (Welch's, Concord, MA), and cinnamon bubble gum (Plen T-Pak Big Red, WM. Wrigley Jr. Co., Chicago, IL).

Enzyme preparation and purification

Rapid isolation of sweet corn germ. Rapid isolation of the germ fraction from sweet corn on the cob was carried out using a modification of the method of Fong and Smith (1985). The precooled fresh sweet corn was husked, inspected, and cut at the top and base of the kernels using a TUC cutter (The United Company. Westminster, MD). The kernels were gently crushed by hand using a rolling pin to release the intact germ and then sieved through three stacked screens with 6.3, 2.36, and 0.83 mm openings. The enriched germ fraction was collected and further isolated by suspending it in ≈35% (w/v) sucrose in a 50 mM sodium phosphate buffer pH 7. All floating tissues were collected and centrifuged in ≈30% (w/v) sucrose in 50 mM sodium phosphate buffer pH 7 at 2,000 × g (4°C) for 5 min to separate germ from the debris. The isolated germ fraction was visually inspected and sorted by hand from non-embryonic tissues before freezing in liquid nitrogen. The frozen isolated germ was stored at −80°C until used.

Preparation of crude enzyme extract. Isolated sweet corn germ was prepared as an acetone powder and extracted with 0.2 M Tris-HCl, pH 8.0 (4°C) as described by Theerakulkait and Barrett (1995b). The supernatant of the crude extract was lyophilized and stored at −23.3°C until needed, at which time it was dissolved in 0.2 M sodium phosphate buffer, pH 7.0, and centrifuged at 17,000 × g for 30 min (4°C). The supernatant was buffer exchanged with 50 mM phosphate buffer, pH 7.0, using a prepacked PD-10 gel filtration column. The extract was frozen in liquid nitrogen and stored at −23.3°C.

Purification of sweet corn germ LPO and POD. LPO in sweet corn germ was purified as described by Theerakulkait and Barrett (1995b). The germ was prepared as an acetone powder, extracted with 0.2M Tris-HCl, pH 8.0 (4°C), fractionated by 40–60% ammonium sulfate saturation, and purified by conventional column chromatography on Sephacryl S-300 HR and the FPLC on a Mono Q column. The pooled active LPO fraction was desalted and buffer exchanged with deionized distilled water using a prepacked PD-10 column, lyophilized, and stored in a desiccator at −23.3°C.

Table 1—Odor descriptors, definitions, reference standards and their preparation and amount used for serving[a] for descriptive sensory evaluation of sweet corn homogenate samples

Descriptors	Reference standards and preparations	Definitions
Overall odor	—	The overall odor impact (intensity) of all compounds perceived by nose.
Painty	Linseed oil: Used 15 mL linseed oil (Grumbacher Artists Oil Medium)	Odor quality associated with the deterioration of the oil fraction. It may be described as linseed oil, paint thinner, shoe polish.
Stale/oxidized	Wet masa harina: Prepared by mixing 1 cup corn tortilla mix (Quaker Masa Harina de Maiz) with 1/2 cup of hot water	Cardboard, old corn flour or the dusty/musty odor that does not include painty.
Cooked cabbage	Sliced cooked cabbage: Prepared by cooking 250 g sliced cabbage with 500 mL of spring water on gas stove at high (10) for 4 min and at low (2) for 30 min; used 10 mL liquid portion and 15 g cooked cabbage	All characteristic notes associated with odor of cooked cabbage, e.g., sour, cabbage, fermented
Straw/hay	Chopped straw and hay: Prepared by chopping the dried straw and hay in a length about 1 to 2 cm; used 3 g chopped straw and 3 g chopped hay	All characteristic notes associated with straw and hay
Corn	Cooked fresh cut sweet corn: Prepared by cooking 75 g fresh cut sweet corn with 5 mL spring water using microwave at full power for 1.5 min; used 30 g cooked cut corn	The characteristic note of "corn" associated with cooked sweet corn
Sweet	Liquid of canned whole kernel corn: Used 30 mL of liquid portion of canned whole corn kernels (Del Monte brand Golden Sweet, Family Style)	The characteristic note of "sweet" associated with canned sweet corn
Cobby/husky	Diced fresh corn cob and fresh corn husk: Prepared by dicing fresh corn husk (thickness about 0.5 cm) and fresh corn cob (thickness about 0.1 cm; used 15 g for diced cob and 8 g for diced husk	The characteristic note associated with diced fresh corn cob and husk

[a] Served in 250 mL clear wine glasses covered with tight fitting aluminum lids.
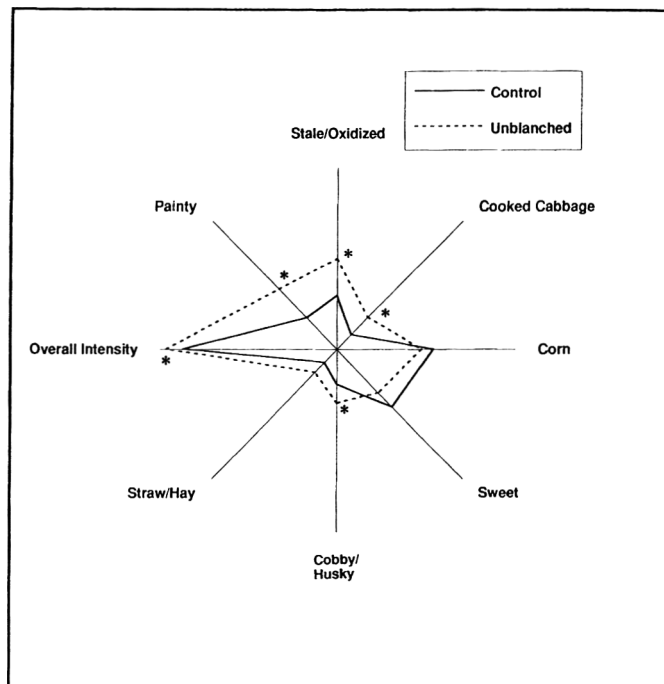
Fig. 1—**Descriptive sensory profile for unblanched sweet corn homogenate samples compared with the control; the distance from the center is the mean value for that odor descriptor.** Means designated with "*" are significantly different from the control at $p < 0.05$.

POD active fractions eluting from both the Sephacryl S-300 HR and the Mono Q columns were collected and those containing at least 30% of the POD activity in the most active fractions from the columns were pooled. The pooled POD fraction was then subjected to further purification by FPLC on a Mono Q column eluted with a linear NaCl gradient. The collected fractions were lyophilized and stored in the desiccator at $-23.3°C$ until used. To prepare the POD for use, it was dissolved in 50 mM sodium phosphate buffer, pH 7.0, and buffer exchanged with phosphate buffer, pH 7.0, using a prepacked PD-10 column.

Enzyme activity assays. LPO activity was determined spectrophotometrically by monitoring the formation of conjugated dienes at 25°C (Theerakulkait and Barrett, 1995a). One unit of enzyme activity is defined as an increase in absorbance of 0.001 at 234 nm/min under assay conditions.

POD activity was determined spectrophotometrically at 470 nm (25°C) by a modification of the procedure of Flurkey and Jen (1978). The substrate solution was prepared by mixing 0.90 mL of guaiacol with $≈180$ mL of 0.2 M sodium phosphate buffer, pH 6.0, for about 20 min, adding 0.02 mL of 30% hydrogen peroxide, and mixing thoroughly. The solution was then adjusted to 200 mL with 0.2 M sodium phosphate buffer, pH 6.0. One unit of enzyme activity was defined as an increase in absorbance of 0.001 at 470 nm/minute under assay conditions.

Protein determination. The protein elution profile was monitored for both conventional column chromatography and FPLC and protein in pooled active fractions was estimated by measuring absorbance at 280 nm. One unit of protein was defined as absorbance of 1.0 at 280 nm.

Sweet corn germ enzymes in off-odor formation

Panel selection and training. A seven-member panel (six females and one male) was selected based on interest, completion of training sessions, availability and consistent performance. Panel training included orientation and development of individual descriptors for the odor of sweet corn homogenate samples. Reference materials were provided to assist with terminology and standardization. Reference standards were anchored at point 3 (30 mL of safflower oil), point 7 (20 mL of orange drink), point 11 (20 mL of grape juice), and point 13 (1 stick of cinnamon bubble gum) and were presented in covered stem glasses. After sufficient training and discussion, the panel agreed on selection of specific odor descriptors. Training was continued until results from the panel and individual panelists were consistent. Final odor descriptors and definitions agreed on by panelists, and reference standards used for each odor descriptor are listed (Table 1).

Odor profile of homogenate of blanched and unblanched frozen stored corn: Sample preparation. Unblanched frozen corn on the cob stored at $-23.3°C$ for 1.75 years and blanched (98°C, 30 min) frozen intact corn kernels stored at $-35°C$ for 1.75 years were used. Samples were prepared by a modification of the procedure described by Velasco et al. (1989). Intact kernels were removed from unblanched, frozen cobs and homogenized in liquid nitrogen using a stainless steel Waring Blendor with the powerstat setting at 100. The liquid nitrogen powder (75g) was weighed into a 450 mL wide mouth freezer jar and allowed to stand at room temperature for about 30 min, after which 15 mL of 50 mM so-
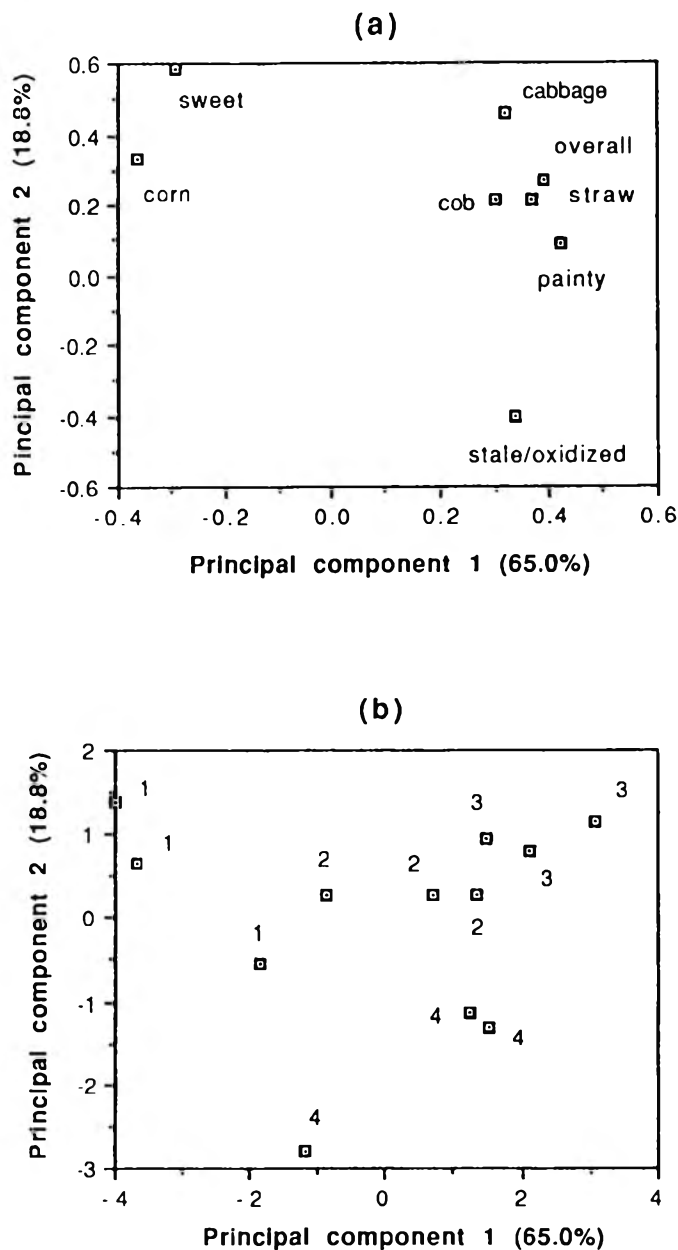
(a)

(b)

Fig. 2—(a) Loading of odor descriptors used for descriptive analysis, and (b) principal component analysis plot of odor intensity rating of odor descriptors for the samples of sweet corn homogenate (1=control, 2=unblanched, 3=added crude enzyme extract and 4=added LPO; each contains three replications) on PC1 vs PC2.
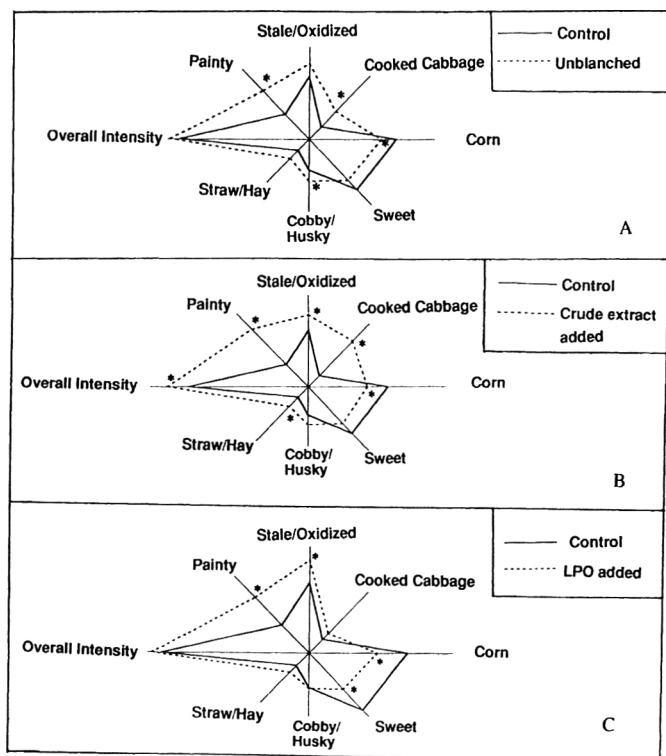

Fig. 3—Descriptive sensory profile for sweet corn homogenate samples (A=unblanched, B=added crude enzyme extract, and C=added LPO; respectively) compared with the control; the distance from the center is the mean value for that odor descriptor. Means designated with "*" are significantly different from the control at p < 0.05.

Sensory profile with addition of purified LPO. Unblanched and blanched frozen intact sweet corn kernels stored at $-35°C$ for 1.75 years were used. Samples included: (1) blanched corn homogenate with added buffer (control), (2) homogenate to which purified LPO was added, (3) homogenate to which crude enzyme extract was added, and (4) unblanched corn homogenate with buffer added. Each of the first three samples were prepared by adding either 50 mM sodium phosphate buffer, pH 7.0, purified LPO solution, or crude enzyme extract in the same phosphate buffer, respectively, to the blanched corn liquid nitrogen powder in a 1 to 5 (v/w) ratio. The unblanched sample was prepared in the same manner as the control. Total LPO activity in the unblanched sample and in the purified LPO and crude extract addition samples was about the same level (675,000 units), while that in the control was insignificant.

Each sample was mixed thoroughly and incubated in a slow speed shaking water bath (30°C) for 3 hr and stirred every 30 min. Samples were heated at 93°C in a water bath for 30 min and then analyzed in the same way, except a set of four (instead of two) samples were used.

Sensory profile with addition of purified POD. Investigation of the involvement of POD in off-odor formation was carried out in a similar manner to the LPO study, except that purified POD was added. Total POD activity in the purified POD and crude enzyme samples was ≈650,000 units.

Statistical analysis. A randomized complete block design was used in all experiments. The block corresponded to each of the seven panelists in each replication. All experiments provided three replications over the treatments using the same panelists. Assessments by panelists were analyzed per odor descriptor through three-way ANOVAs with panelist (P), replication (R) and treatment (T) as factors. The interactions for each descriptor were also tested for significance. SAS version 6 (SAS Institute, Inc., 1987) was used for statistical analysis. A mixed effect linear model was used with panelist and replication as random effects (Lundahl and McDaniel, 1988), while treatment was considered a fixed effect. For the model containing all 2- and 3-factor interactions, the F-statistic for testing treatments (F,) was calculated according to Steele and Torrie (1980) by the following formula:

$$F_t = MS(T) + MS(P \cdot R \cdot T)$$

$$MS(R \cdot T) + MS(P \cdot T)$$

However, since the replication-by-treatment interactions (R·T) for all

dium phosphate buffer, pH 7.0, were added. The control sample was prepared in the same manner, except the homogenate of blanched frozen corn kernel was used. Each sample was mixed thoroughly, covered with a watch glass, and heated at 93°C in a water bath for 30 min, stirring every 10 min. Each sample (8g) was weighed into 250 mL black, tulip-shaped stem glasses coded with three digit random numbers, capped with aluminum lids and sealed with parafilm.

Sample testing. Samples were cooled by leaving them at room temperature (22°C) for at least 30 min. A set of two samples was presented in random order to each panelist in individual booths. Panelists were asked to remove the cover, sniff the head space odor and rate the intensity of each odor descriptor. Panelists sniffed water after each sample to avoid minimize cross-over effects.

Each odor descriptor intensity was rated using a 16-point intensity scale, (0=none, 1=just detectable, 3=slight, 5=slight to moderate, 7=moderate, 9=moderate to large, 11=large, 13=large to extreme, and 15=extreme). Reference standards for each odor descriptor and for odor intensity were available for panelists to review in every session. The testing was performed in 3 individual sessions (3 replications).

Descriptors	Treatments				LSD (p ≤ 0.05)	Sig.
	Control	Unblanched	Crude extract	LPO		
Overall intensity	9.09[b] (1.22)	9.81[b] (1.29)	10.71[a] (1.35)	9.71[b] (1.68)	0.84	••
Painty	2.38[b] (1.99)	4.62[a] (3.54)	6.10[a] (2.81)	4.71[a] (3.20)	2.02	••
Stale/Oxidized	4.24[b] (1.81)	5.14[ab] (1.35)	5.38[a] (1.60)	5.62[a] (1.53)	1.06	p = .06
Cooked cabbage	1.19[c] (1.97)	2.67[b] (2.39)	4.86[a] (2.95)	1.71[bc] (1.95)	1.41	•••
Straw/Hay	1.10[b] (1.67)	1.86[ab] (1.82)	2.05[a] (2.40)	1.67[ab] (1.83)	0.79	p = .09
Corn	6.24[a] (1.22)	5.14[b] (1.32)	4.62[b] (1.80)	4.33[b] (1.35)	0.95	••
Sweet	4.86[a] (1.88)	3.95[ab] (1.75)	3.86[ab] (1.77)	3.10[b] (1.48)	1.15	•
Cobby/Husky	2.10[b] (1.61)	2.86[a] (1.49)	2.81[ab] (1.94)	2.1[a][ab] (1.56)	0.79	p = .08

[z] Sixteen point intensity scale (0 = none, 15 = extreme).

[a-c] Means with the same letter, in the same row, are not significantly different at the 0.05 significance level by LSD

descriptors in all experiments were not statistically significant, the appropriate F-statistic was simplified to:

$$F_t = \frac{MS(T)}{MS(P^*T)}$$

The mean square for panelist-by-treatment interaction (P*T) was used as the error term for the test for treatment effect. Comparisons of treatment means of each odor descriptor were conducted using Fisher's least significant difference test (p ≤ 0.05).

Data were also analyzed by principal component analysis (PCA) using SAS (SAS Institute, Inc., 1987). The PCA scores for each axis were analyzed by ANOVA and by Fisher's least significant difference test (p ≤ 0.05).

RESULTS & DISCUSSION

Purification of sweet corn germ LPO and POD

In order to study involvement of sweet corn germ enzymes in off-odor formation, a crude enzyme extract and purified LPO and POD extracts were prepared. The purification of LPO was 188 fold with 26.3% recovery. The majority of the POD eluted from the column later than LPO, indicating that the molecular size of the major POD isozymes in sweet corn germ was smaller than that of LPO. On the FPLC Mono Q column, the majority of POD eluted from the column before starting the NaCl gradient both during purification of LPO and POD, indicating that the majority of POD was in the basic form. The purification and recovery of POD in pooled fraction was 58 fold and 27.6%, respectively. The purification scheme for both enzymes was taken to this point because both POD and LPO were free of activity of the other enzyme.

Sweet corn germ enzymes in off-odor formation

Odor profile of homogenate of blanched and unblanched frozen stored corn. Prior to investigating the involvement of sweet corn germ enzymes in off-odor formation, the profile of a homogenate of blanched frozen intact corn (control) was evaluated. The odor profile of the control was described as slight to moderate "sweet" and "corn," just detectable to slight "cobby/husky," "painty", and "stale/oxidized", and just detectable "straw/hay", and "cooked cabbage" with moderate to large "overall odor intensity" (Fig. 1). The overall odor characteristics were relatively high in the "desirable" odors of sweet corn including "sweet" and "corn", and relatively low in "undesirable" or "off-odor" characteristics, including "stale/oxidized," "painty," "cobby/husky," "cooked cabbage" and "straw/hay" descriptors.

The odor profile of homogenate prepared from frozen stored raw (unblanched) corn on the cob was compared with the control using a typical descriptive sensory profile (Stone et al., 1974, Fig. 1). Univariate ANOVA on each descriptor showed significant treatment differences between unblanched and control samples for most of the off-odor descriptors except for "straw/hay" and "desirable" odor descriptors "sweet" and "corn." This implied that differences between samples were significantly detectable by the trained panel for most descriptors of off-odor characteristics. Replication effects were not significant for any descriptors except "painty," indicating good reproducibility of replicates.

When both treatment and panelist by treatment interactions are significant, it is important to examine whether panelist by treatment interaction influences conclusions regarding treatments. This was done by comparing the line graph plot between each panelist's ratings for each treatment to search for systematic inconsistencies among the panelists contributing to variation. For example, in the case of "stale/oxidized" there was a significant effect of both treatment and panelist-by-treatment interaction. It was found that most of the panelists responded similarly, suggesting that main treatment effect differences were important.

The mean odor intensity of the homogenate of unblanched sample was higher than the control in "overall intensity" and in most off-odor descriptors, while the mean intensity of "desirable" odor descriptors not different from the control. Unblanched corn on the cob developed off-odor during frozen storage as indicated by the off-odor characteristics of its homogenate. The development of off-flavor and off-odor in frozen raw (unblanched) or underblanched sweet corn stored in freezers for extended times has also been reported for cooked corn on the cob (Wagenknecht, 1959; Lee, 1981) or cooked whole kernels (McDaniel et al., 1988).

Sensory profile with addition of purified LPO. The effects of addition of crude enzyme extract and purified sweet corn germ LPO on off-odor formation were studied. Only the first two principal components (PC) were significant (Fig. 2) with 64.99 and 18.81% of total variation explained by PC1 and PC2, respectively. The loading of odor descriptors for PC1 and PC2 indicated that PC1 could be defined as a "desirable" vs. "undesirable" (off-odor) descriptor axis (Fig. 2a). The overall intensity and off-odor descriptors, especially "painty," negatively correlated with "desirable" odor descriptors, especially "corn." PC2 might be defined as "stale/oxidized" vs "sweet" and "cooked cabbage."

ANOVA tests indicated that there were significant differences between the mean PC1 and PC2 scores among samples, with
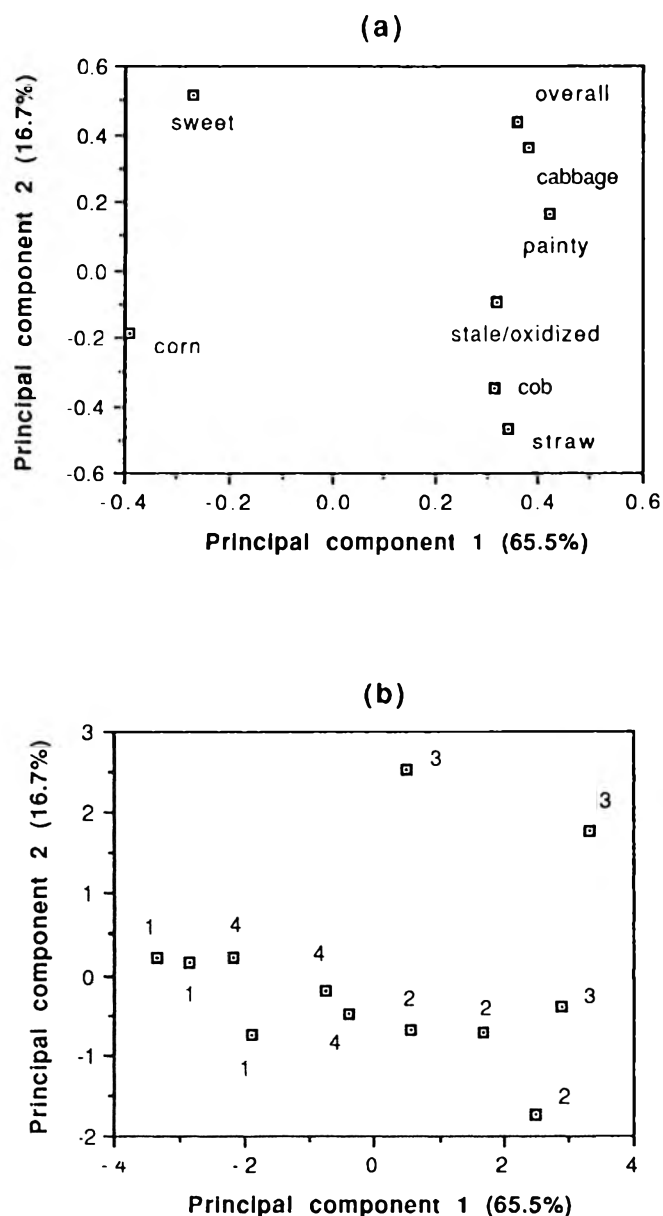
(a)

(b)

Fig. 4—(a) Loading of odor descriptors used for descriptive analysis, and (b) Principal component analysis plot of odor intensity rating of odor descriptors for the samples of sweet corn homogenate (1=control, 2=unblanched, 3=added crude enzyme extract and 4=added POD; each contains three replications) on PC1 vs PC2.

non-significant effects of replications indicati: a good reproducibility. This was also observed from the grouping of 3 replications for each sample (Fig. 2b). The PCA plot of odor intensity ratings indicated that the samples could be classified into 3 groups (Fig. 2b): (1) control, unblanched and added crude extract (2 and 3), and added purified LPO (4). The mean PC1 score of the control was different from that of unblanched, added crude extract, and added purified LPO samples, which were not different from each other. The control was best described by "desirable" odor descriptors while the other samples were described by off-odor descriptors (Fig. 2a and b). However, the mean PC2 score of the sample with added purified LPO was different from those of unblanched and added crude extract. The sample with added purified LPO seemed to be best described by "stale/oxidized" and "painty" compared to the other samples (Fig. 2a and b).

The results of univariate ANOVA on each descriptor showed treatment differences among samples for "cooked cabbage," "overall intensity," "painty," "corn," and "sweet." A pan-

elist effect was significant for all odor descriptors; however, this was not unusual and reflects some panelists' usage of different portions of the intensity scale (Power, 1988). The replication effect was not significant for any odor descriptors, indicating good reproducibility. Panelist by treatment interaction effects were significant for "painty," "sweet," "corn" and "cobby/husky;" however, most of panelists responded similarly.

The mean values of odor intensity of most off-odor descriptors including "painty," "cooked cabbage," and "cobby/husky" of the unblanched sample were higher, while the descriptor "corn" was lower than that of the control. The odor profile of the unblanched sample incubated at 30°C for 3 hr seemed to be similar to that of the unblanched sample frozen stored at -23.3°C for 1.75 years (Fig. 1 and Fig. 3).

The crude enzyme extract sample was higher in intensity of most off-odor descriptors including "painty," "stale/oxidized," "cooked cabbage," "straw/hay" than that of the control, but the "corn" odor was lower (Fig. 3). This sample, like the unblanched one, showed a higher mean intensity of off-odor descriptors, and a lower intensity of "desirable" odor descriptors than the control. However, the mean intensity of descriptors "overall intensity" and "cooked cabbage" of the crude extract was also higher than that of the unblanched and added LPO samples (Table 2).

Addition of purified LPO to the homogenate resulted in an increase in off-odor descriptors "painty" and "stale/oxidized," and a decrease in "desirable" odor descriptors "sweet" and "corn" (Fig. 3). The decrease in "sweet" and "corn" odors may be due to masking by the increase in intensity of those off-odor descriptors. The formation of typical cooked "corn" odor is generally hypothesized to be heat activated, while raw sweet corn has very little odor. Dimethyl sulfide (DMS) is one of the principle low-boiling volatile compounds that contributes to cooked "corn" odor (Bills and Keenan, 1968; Williams and Nelson, 1973; Flora and Wiley, 1974; Dignan and Wiley, 1976; Wiley, 1985; Azanza et al., 1994).

Azanza et al (1994) found no association between DMS concentration and raw sweet corn odor, but found that grassy odor and flavor scores correlated with DMS concentration. They found that sweet corn samples with high DMS concentrations also had high concentrations of other volatiles which may contribute to grassy odor and flavor. LPO activity was not analyzed, therefore they could not determine whether enzyme-catalyzed production of hexanal and other short chain alcohols was responsible for grassy odor or flavor.

The observed increases in the off-odor descriptors "painty" and "stale/oxidized" in the added purified LPO and crude extract samples may be caused by LPO-catalyzed hydroxyperoxidation of polyunsaturated fatty acids and esters containing a cis, cis-1,4-pentadiene system. This reaction initially yields hydroperoxides which subsequently degrade to form a variety of secondary products, including aldehydes, alcohols, and ketones, which may result in off-odor formation (Eskin et al., 1977; MacLeod and Ames, 1988). Wagenknecht (1959) and Lee (1981) suggested that enzymes, particularly LPO, induced off-flavors in unblanched sweet corn.

Previous studies indicated that LPO was important in off-flavor and off-odor formation. McDaniel et al. (1988) reported that mean intensity of the descriptor "stale/oxidized" was higher in underblanched frozen stored corn than in commercially blanched frozen stored corn. Kalbrener et al. (1974) reported a "musty/stale" odor was a predominant descriptor of the linoleic hydroperoxide produced by soy LPO oxidation of linoleic acid. Other investigators (Ashraf and Synder, 1981; Johnsen et al., 1988; Civille and Dus, 1992; Mistry and Min, 1992) have reported "painty" and "stale/oxidized" off-odors in soy milk, peanut and vegetable oil, and other products in which LPO is present. Moreover, LPO was the key enzyme involved in off-odor formation in English green peas and green beans (Williams et al., 1986).

Table 3—Mean ratings[z], standard deviations (SD), LSD values for odor descriptors among the treatments for POD study

| Descriptors | Treatments | | | | LSD (p ≤ 0.05) | Sig. |
	Control	Unblanched	Crude extract	POD		
Overall intensity	8.67[c] (0.97)	9.76[ab] (1.55)	10.57[a] (1.40)	8.95[bc] (1.32)	0.90	**
Painty	1.57[c] (1.96)	4.43[ab] (3.44)	5.57[a] (2.34)	2.57[bc] (2.50)	1.96	**
Stale/Oxidized	3.33[a] (2.08)	4.33[a] (1.96)	4.29[a] (2.55)	4.29[a] (2.35)	1.58	ns
Cooked cabbage	1.05[c] (1.66)	2.90[b] (2.66)	4.86[a] (2.65)	1.48[bc] (1.94)	1.69	***
Straw/Hay	1.05[b] (1.32)	2.00[a] (1.76)	1.52[ab] (1.81)	1.29[b] (1.55)	0.48	**
Corn	6.19[a] (1.36)	4.90[b] (1.64)	3.90[bc] (1.95)	5.29[ab] (1.38)	1.16	**
Sweet	4.71[a] (2.39)	3.48[a] (1.63)	4.05[a] (2.80)	4.38[a] (2.18)	1.43	ns
Cobby/Husky	2.33[a] (2.11)	3.05[a] (1.63)	2.62[a] (1.91)	2.29[a] (1.90)	1.04	ns

[z] Sixteen point intensity scale (0 = none, 15 = extreme).

[a-c] Means with the same letter, in the same row, are not significantly different at the 0.05 significance level.

*,**,*** refers to significance at p ≤ 0.05, 0.01 and 0.001, respectively by ANOVA F-test. ns = not-significant at p ≤ 0.05.

Although lipoxygenase appears to be a primary cause, other sweet corn germ enzymes may be involved in formation of other off-odor descriptors, particularly "cooked cabbage," which was higher in the sample with added crude extract than that with added purified LPO. Gardner (1970) reported that linoleate hydroperoxide isomerase was present in the germ of mature corn and that it catalyzed the production of hydroperoxide products in addition to LPO. Velasco et al. (1989) also hypothesized that LPO was important in off-odor development in sweet corn, however; they suggested that other enzymes, such as hydroperoxide isomerase and hydroperoxide lyase, may be important.

Sensory profile with addition of purified POD. In terms of POD involvement in off-odor formation, the first two PC explained most of the total variation, with 78.53 and 13.58% for PC1 and PC2, respectively. As with the LPO experiment, PC1 may be defined as a "desirable" and "off-odor" descriptor axis (Fig. 4a). However, mean PC scores among the samples were different only for PC1. The replication effect was not significant in either case indicating good repeatability.

Based on the LSD test of means in PC1, the samples could be classified into two groups: control and added purified POD samples, and unblanched and added crude extract samples. The mean PC1 score of the added purified POD sample was not different from that of the control, but was different from those of unblanched and added crude extract samples. However, the unblanched and added crude extract samples were not different from each other. The control and added purified POD samples were similar in odor profile and could be best described by "desirable" odor descriptors "sweet" and "corn", while the added crude extract and unblanched samples were best described by off-odor descriptors (Fig. 4a, b).

The results of univariate ANOVA on each descriptor showed treatment differences among samples for "cooked cabbage," "overall intensity," "painty," "straw and hay," and "corn." The experiment showed good reproducibility as indicated by the non-significant replication effect. The panelist by treatment interaction was significant for most descriptors except "straw/hay" and most of the panelists responded similarly. Mean intensity of most off-odor descriptors for the unblanched sample, including "painty," "cooked cabbage," and "straw/hay," were higher than the control, but "corn" odor was lower than that of the control (Table 3). This result was similar to that observed for the unblanched sample from the LPO study.

The mean values of intensity for the added crude enzyme extract sample were higher than the control in "overall intensity" and the off-odor descriptors "painty" and "cooked cabbage." The "corn" odor was lower than that of the control (Fig.
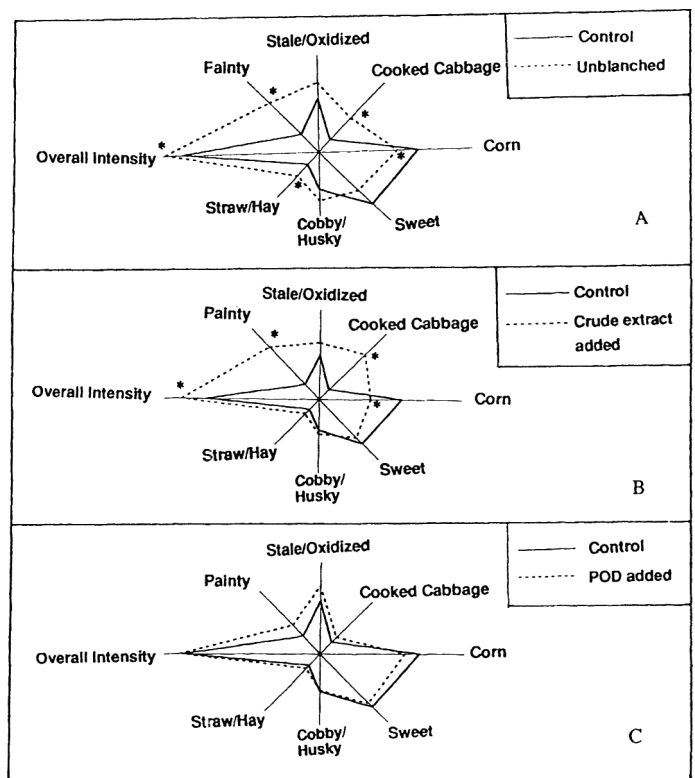

Fig. 5—Descriptive sensory profile for sweet corn homogenate samples (A=unblanched, B=added crude enzyme extract, and C=added POD; respectively) compared with the control; the distance from the center is the mean value for that odor descriptor. Means designated with "*" are significantly different from the control at p < 0.05.

5). The odor profile of the sample with added crude enzyme extract was similar to that of unblanched sample. The mean intensity of descriptors describing off-odor was higher than the control while that of descriptors describing "desirable" odor was lower than that of the control. However, the mean intensity of descriptor "cooked cabbage" was higher than that of the unblanched sample (Table 3).

The mean values for intensity of all odor descriptors for the added POD sample were not different from that of the control. However, adding crude extract, which contained the same total POD activity, resulted in increases in mean intensity of off-odor

descriptors "painty" and "cooked cabbage" and a decrease in mean intensity of desirable-odor descriptor "corn." These results suggests that POD presence in sweet corn germ was not an important factor in off-odor formation, especially in the formation of "painty" and "cooked cabbage" odors. Other investigators (Williams et al., 1986; Lim et al., 1989; Velasco et al., 1989) have suggested that POD was not important in off-odor formation in plant tissues.

CONCLUSIONS

LPO IN SWEET CORN GERM is important in off-odor formation, particularly in production of odors described as "painty" and "stale/oxidized." POD presence in sweet corn germ does not appear to affect off-odor. Other enzymes in the germ may also be involved in off-odor formation, especially the "cooked cabbage" odor. Results suggest that analysis of LPO activity, rather than POD, may be a more appropriate index of blanching adequacy.

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Ms received 7/18/94; revised 3/8/95; accepted 4/1/95.

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Peroxidase and Lipoxygenase Influence on Stability of Polyunsaturated Fatty Acids in Sweet Corn (*Zea mays* L.) during Frozen Storage

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ABSTRACT

The effect of blanching treatments and packaging materials on lipoxygenase (LOX) and peroxidase (POD) activity and fatty acid stability of two cultivars of sweet corn (Jubilee and GH-2684) were evaluated during 9 mo storage at −20°C. Complete inactivation of LOX and POD was obtained with 9 and 15 min of steam blanching, respectively. Relative fatty acid content revealed no change in fatty acid composition during storage. Control of degradation of polyunsaturated fatty acids (PUFA) did not depend on oxygen permeability of different packaging materials. Blanching had little effect on PUFA degradation after 9 mo storage.

Key Words: sweet corn, peroxidase, lipoxygenase, polyunsaturated, fatty acids

INTRODUCTION

LIPID OXIDATION is a major cause of food spoilage because it leads to development of off-flavors and odors in edible oils and fat-containing foods (Nawar, 1985). The oxidative deterioration of food lipids involves primarily autoxidation by free radical chain reaction, photo-oxidation (Frankel, 1980; Nawar, 1985) and enzymatic oxidation catalyzed by lipoxygenases (Siedow, 1991; Galliard and Chan, 1980; Hildebrand, 1989). The primary initial products of lipid oxidation are hydroperoxides which decompose into a wide range of carbonyl compounds, hydrocarbons, aldehydes, ketones and other materials causing rancidity (Frankel, 1991).

Freezing is used to maintain product quality over long storage and results in a slower rate of most deteriorative reactions such as senescence, enzymatic decay, chemical decay and microbial growth (Labuza, 1982). However, freezing does not prevent off-flavor development, color and texture deterioration in frozen vegetables because enzyme systems may remain active even at sub-zero temperatures. Blanching, before freezing, is used to inactivate enzymes, reduce microorganisms and increase digestibility of some products. It also improves color and flavor of vegetables as a result of removal of gases. Adverse effects of blanching are permanent modification of cellular structure, solubilization and/or destruction of some nutrients and vitamins, and conversion of green chlorophylls to yellow green pheophytins (Bald, 1991; Katsaboxakis, 1984).

To minimize thermally induced textural changes or nutrient leaching, it has been suggested that blanching be optimized for each product. This requires determining the heat treatment needed to inactivate enzymes responsible for deleterious changes during freezing and frozen storage (Reid, 1990; Williams et al., 1986).

POD is widely used to indicate adequate blanching because it is highly resistant to heat inactivation, is present in most vegetables and fruits. It is detected by a sensitive and simple colorimetric test (Richardson and Hyslop, 1985). Correlations have been reported between off-flavor development and POD activity

in frozen sweet corn on the cob (Lee and Hammes, 1979). POD, like most heme proteins, catalyzes the nonenzymatic, peroxidative degradation of unsaturated fatty acids yielding volatile and flavorful carbonyl compounds that may contribute to oxidized flavor (Richardson and Hyslop, 1985). However, some studies have shown that POD is not directly involved in quality deterioration of frozen unblanched vegetables and its complete inactivation may result in overblanching (Williams et al., 1986). The measure of LOX activity has been suggested as an indicator of adequacy of blanching for several vegetables (Axelrod et al., 1981; Sheu and Chen, 1991; Chen and Hwang, 1988; Williams et al., 1986; Chen and Whitaker, 1986; Wagenknecht and Lee, 1958) including sweet corn (Garrote et al., 1985; Wagenknecht, 1959). It is closely related to destruction of essential fatty acids, off-flavor development and pigment degradation.

Our objective was to evaluate the relationship between LOX and POD activities with polyunsaturated fatty acid stability in two cultivars of sweet corn on the cob (Jubilee and GH 2684). They were subjected to different blanching treatments and stored using different packaging materials for 9 mo at −20°C.

MATERIALS & METHODS

Plant material

Fresh sweet corn (*Zea mays* L.) of Jubilee and GH-2684 (Rogers NK seed Co, Research Center) cultivars were obtained from the Oregon State University Vegetable Experimental Station. Both were harvested on the same day, using the percent moisture content of kernels as an index of maturity.

Processing of samples

Sweet corn (≈1000 ears) of each variety were harvested and immediately transported to the Oregon State University Food Science & Technology Pilot Plant where they were randomly divided into three lots (replicates). Each lot was husked, randomly divided into three groups of 39 ears each and processed in two batches. One group served as control and the other two were steam blanched at 100°C for 9 and 15 min, respectively. After blanching, the sweet corn ears were cooled immediately in water (8°C) for 25 min, drained and frozen on trays in a blast freezer at −35°C for 2 hr. The frozen ears from each treatment group were cut at each end to provide ears of ≈14 cm length. Three ears were used for zero time analysis and the remaining ears were divided into four subgroups of nine ears each and packed using polyethylene, Cryovac E-Bag (E bags), Cryovac Barrier Bag (B bags) (Cryovac Division, W.R. Grace & Co., Duncan, SC) or no packing material (control). Cryovac E and Cryovac B bags have moisture permeabilities of 0.1 g/100 cm²/24 hr at 100% relative humidity and 38°C. They differed in oxygen permeability (40 mL/100 cm²/24 hr at 23°C and 1 atm for E-bags and 0.3-0.4 mL/100 cm²/24 hr at 23°C and 1 atm for B-bags, Deak et al., 1987). Ears were placed three to a pack for each packing material. The polyethylene bags were hand sealed and the Cryovac B and E bags were heat sealed using a Verwachting vacuum sealer. The ears were stored for 9 mo and analyses were performed at 0, 3, 6 and 9 mo.

Moisture analysis

The moisture content was determined using the microwave method of Beckwar et al. (1977). Analyses were performed before processing and after 9 mo frozen storage at −20°C.

The moisture content on fresh corn was determined from samples composed of kernels pooled from six separate ears of corn. A total of 18 ears were used for analysis (triplicate analysis). Moisture analyses were done at 9 mo on samples prepared from kernels pooled from three separate ears of corn packed under specific packaging materials. Nine ears of corn were used to prepare three samples, with two of the samples being analyzed.

Sample preparation

Whole kernels of sweet corn (100 g) were frozen in liquid nitrogen and blended using a Waring Blendor at 100 rpm for 1 min. In order to keep the sample frozen for efficient blending, liquid nitrogen was added every 15 sec. A fine powder was obtained which was stored in a cold container at −20°C until analyzed.

Enzyme assays

Enzyme extraction. The powdered corn (2 g) was placed in a centrifuge tube and 20 mL of 0.1M Tris-HCl buffer pH 8.0 were added. The mixture was homogenized using a tissuemizer at 50 rpm for 3 min. After homogenization, the probe was rinsed with 1 mL of 0.1M Tris-HCl buffer pH 8.0. The homogenate was centrifuged using a Sorval RC5 superspeed refrigerated centrifuge in an SS 34 rotor at 12,000 rpm (17210 × g) for 1 hr at 4°C. The supernatant was removed, avoiding the floating top layer, and used for activity measurements.

Lipoxygenase activity. Lipoxygenase (linoleate:oxygen oxidoreductase, EC 1.13.11.12) was assayed by the method of Chen and Whitaker (1986), based on absorption at 234 nm of the conjugated dienes formed when linoleic acid (used as substrate) was oxidized in the presence of LOX. The substrate solution was prepared by mixing 157.2 μL linoleic acid, 157.2 μL Tween 20 and 10 mL distilled water, clarified by adding 1 mL of 1.0N NaOH and diluted with 0.1M sodium phosphate buffer pH 7.0 to a final volume of 200 mL. Prior to assay, the substrate solution was transferred to an amber container, aerated with oxygen for 10 min and allowed to stand in a shaking water bath (American YB-521) at 25°C for 10 min.

For the assay, 150 μL of enzyme extract was added to 2.85 mL of linoleic acid substrate solution in a quartz cuvette and mixed. The LOX activity was measured from the change in absorbance at 234 nm over time using a Shimadzu 160U UV-VIS spectrophotometer and the temperature was controlled at 25°C. One unit of enzyme activity was defined as that amount of protein that produced a change of 1 unit in absorbance/min.

Peroxidase activity. Peroxidase was assayed by the method of Sheu and Chen (1991), based on the increase in absorbance at 420 nm resulting from oxidation of guaiacol in the presence of hydrogen peroxide. The substrate solution was prepared by mixing 558 μL guaiacol and 194.4 μL of 30% hydrogen peroxide; the solution was then diluted to 100 mL with 0.2M sodium phosphate buffer pH 6.0 to give a concentration of 0.05M guaiacol and 0.2M hydrogen peroxide. POD activity was measured from initial increase in absorbance at 420 nm over time with a Shimadzu 160U UV-VIS recording spectrophotometer and the temperature was controlled at 25°C. One unit of enzyme activity was defined as that amount of protein that produced a change of one unit in absorbance/min.

Protein

Protein was assayed using the method of Lowry et al. (1951) by measuring absorbance at 700 nm. Bovine serum albumin was used as standard.

Fatty acids analysis

Corn lipids were extracted following the procedures proposed by Bligh and Dyer (1958). Fatty acids were analyzed by the fatty acyl methyl esters (FAME) method (Selivonchick, 1977). The lipid extracted was transesterified by heating in 4% methanolic H_2SO_4 solution at 80–90°C for 90 min. A partition of hexane and water was used to extract the methyl esters. The hexane phase was collected and evaporated under nitrogen and the dried sample was taken up into 0.5 mL iso-octane, flushed with nitrogen and stored at −80°C until analyzed by gas chromatography.

Analysis of methyl esters was performed on a Hewlet Packard 5890 Gas Chromatograph equipped with FID, 3393A Integrator and 9122 Dual Disc Drive. A 30m × 0.25 mm i.d. fused silica Supelco 2330 capillary column was used for analysis of all nitrogen powdered corn

fatty acid methyl esters. Conditions were: injection port and detector 220°C, the column was run isothermally at 175°C for 10 min and increased to 210°C at 5°C/min with a final hold time of 10 min. The carrier gas was helium at 1 mL/min. Fatty acid methyl esters were identified by comparing to authentic standards Nu Check 20A (Nu Check Prep) and corn oil fatty acids.

Determination of peroxide value

The peroxide value was determined using the method of Schmedes and Holmer (1989). The method is based on the oxidation of Fe(II) to Fe(III) by peroxides; Fe(III) forms a violet complex with thiocyanate and this complex is quantitated spectrophotometrically. For calculation of peroxide value, the absorbance of the sample and lipid blank were converted to mg Fe(III)/5 mL solution using standard curve values and the net value in terms of mg of Fe(III)/5 mL of solvent was calculated by the difference between values obtained for the sample and blank.

The peroxide value was expressed in terms of meq oxygen/kg lipid (Stine et al., 1954).

Statistical analysis

The experiment was conducted using a split-plot design. The split occurred at the blanching stage, with variety and blanching as whole plot and packaging material and storage time as sub-plot. The data were evaluated using Statistical Analysis System (SAS Institute, Inc. software). Data were further evaluated by a regression analysis using the stepwise procedure (Ramsey and Schafer, 1992). The regression analyses were performed using the Statistical Graphics System (Stat-Graphics software).

RESULTS & DISCUSSION

Lipoxygenase activity

There was substantial evidence that the initial level of LOX activity in the unblanched whole kernels varied between the cultivars (2-sided p-value < 0.0001, step backward regression analysis). The Jubilee showed an initial mean LOX activity of 0.143 ± 0.032 units/mg protein, 32.4% higher than the mean LOX activity of the GH-2684 with a level of 0.108 ± 0.017 units/mg protein. It has been reported that LOX activity varied among cultivars, different organs within the same plant (Garrote et al., 1985; Vick and Zimmerman, 1976), and development stages (Kermasha and Metche, 1987). The regression analysis indicated that LOX activity decreased during the 9 mo of frozen storage in both unblanched sweet corn cultivars, even after accounting for the effects of different cultivars (2 sided p-value < 0.0001). Steam blanching at 100°C for 9 min completely inactivated LOX activity in both cultivars. Blanching for 15 min had no further effect on LOX activity (Table 1).

Peroxidase activity

No significant difference in initial POD activities were found in kernels of any unblanched sweet corn (Table 1). An average activity of 1.082 ± 0.039 and 1.075 ± 0.051 units/mg protein was determined for Jubilee and GH 2684 respectively. Regression analysis indicated (2 sided p-value < 0.0001) that the POD activity of unblanched samples decreased as a quadratic function of frozen storage time. The POD activity decreased sharply in the first 3 mo with a tendency to stabilize during later frozen storage. Both cultivars showed similar changes during 9 mos frozen storage (Table 1).

After 9 min of blanching some residual POD activity was present. A regression analysis for the 9 min blanched samples over storage time showed that the POD activity had a tendency to decrease faster in the GH 2684 than in the Jubilee (2 sided p-value < 0.0001), suggesting the presence of POD isozymes that were more stable to low temperatures in the Jubilee. Complete inactivation of POD activity was obtained when the corn was blanched for 15 min.

The data showed a decrease in activity of LOX and POD during frozen storage. According to Richardson and Hyslop

Table 1—Lipoxygenase (LOX) and Peroxidase (POD) activity in whole kernels of Jubilee and GH-2684 corn during 9 mo storage at -20°C[a]

Blanching time (min)	LOX activity (change in O.D. at 234 nm/min/mg protein)				POD activity (change in O.D. at 420 nm/min/mg protein)			
	Storage time (months)				Storage time (months)			
	0	3	6	9	0	3	6	9
Jubilee								
0	0.143	0.105	0.108	0.087	1.082	0.450	0.575	0.476
	(0.016)	(0.016)	(0.017)	(0.016)	(0.019)	(0.024)	(0.034)	(0.039)
9	N.A.[b]	N.A.	N.A.	N.A.	0.048	0.032	0.048	0.030
					(0.015)	(0.016)	(0.017)	(0.010)
15	N.A.[b]	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.
GH-2684								
0	0.108	0.102	0.094	0.062	1.075	0.493	0.541	0.453
	(0.017)	(0.014)	(0.016)	(0.010)	(0.025)	(0.015)	(0.061)	(0.030)
9	N.A.	N.A.	N.A.	N.A.	0.081	0.033	0.036	0.025
					(0.027)	(0.013)	(0.015)	(0.009)
15	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.

[a]Averages over packaging materials; no significant differences observed among them (2-sided p-value > 0.05). ( ): Standard deviation.
[b] N.A.: no activity detected.

Table 2—Relative percent linoleic and linolenic acid in whole kernels of the Jubilee and GH-2684 corn during 9 mo storage at -20°C[a]

Blanching time (min)	Relative percent linoleic acid content (%)				Relative percent linolenic acid content (%)			
	Storage time (months)				Storage time (months)			
	0	3	6	9	0	3	6	9
Jubilee								
0	44.73	46.97	43.48	43.42	1.82	2.01	1.79	1.50
	(2.57)	(1.70)	(3.81)	(2.55)	(0.43)	(0.19)	(0.32)	(0.26)
9	49.29	46.64	45.29	44.37	2.07	2.06	1.90	1.62
	(2.15)	(2.36)	(1.47)	(4.11)	(0.13)	(0.21)	(0.27)	(0.44)
15	45.65	46.50	44.27	44.67	1.97	1.88	1.84	1.64
	(0.33)	(0.93)	(4.22)	(1.92)	(0.25)	(0.08)	(0.42)	(0.19)
GH-2634								
0	48.82	45.76	43.78	44.01	1.91	1.75	1.33	1.03
	(1.44)	(1.74)	(4.32)	(1.01)	(0.25)	(0.23)	(0.39)	(0.14)
9	45.50	45.91	45.29	44.70	1.82	1.57	1.68	1.28
	(6.03)	(2.52)	(2.50)	(1.44)	(0.09)	(0.18)	(0.25)	(0.18)
15	47.23	46.83	43.94	45.16	1.77	1.58	1.40	1.17
	(0.73)	(1.28)	(3.27)	(0.94)	(0.04)	(0.14)	(0.29)	(0.08)

[a]Averages over different packaging materials; no statistical differences observed among them (2-sided p-value > 0.05). ( ): Standard deviation.
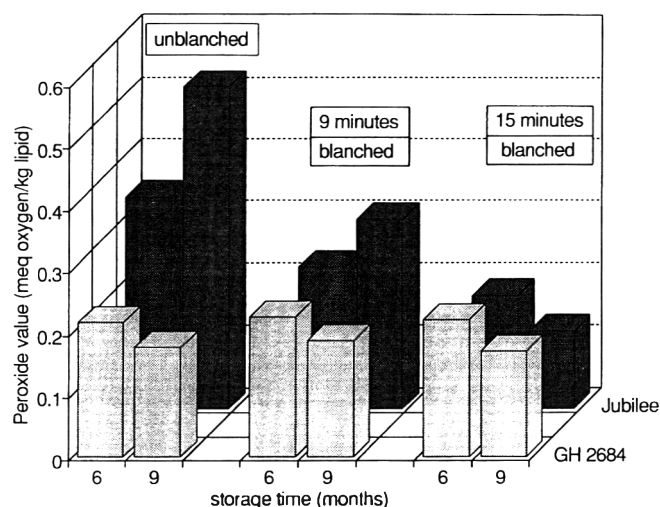

Fig. 1—Change in peroxide value, (meq O₂/kg lipid), in whole kernels of Jubilee and GH-2684 corn during sixth and ninth months of storage at -20°C. Averages over different packaging materials; no statistical differences observed among them (2-sided p-value > 0.05).

Fig. 2—Average moisture content of sweet corn kernels of Jubilee and GH-2684 after 9 mo storage at -20°C. Packaging materials significantly different at p < 0.05.

(1985) the decrease in enzyme activity during freezing and/or frozen storage may be due to a change in stability of enzyme conformers, increased intra-enzymic hydrogen bonding or decreased accessibility of enzyme to substrate. It could also be due to increased hydrogen bonding between water and either substrate or enzyme active site, formation of enzyme polymers, changes in mechanism, shifts in pH or increases in viscosity.
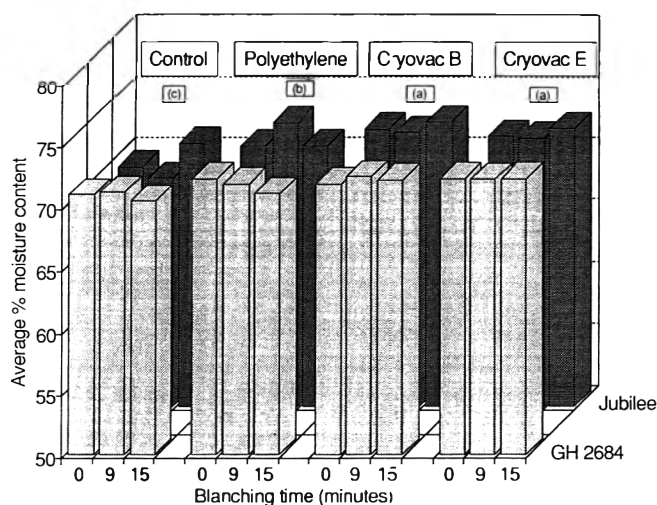
Fatty acid composition

Although the corn evaluated had different gentoypes, no significant difference in initial fatty acid content was observed. The major fatty acids were palmitic (14.93 ± 1.94%), stearic (2.79 ± 1.00%), oleic (31.54 ± 2.82%), linoleic (46.87 ± 5.88%), and linolenic (1.89 ± 0.36%) acids. There was no statistical difference in relative percent linoleic acid between the cultivars. Different blanching treatments had no effect on relative percent linoleic acid content, suggesting that enzymatic oxidation of lin-

oleic acid did not occur during frozen storage. There was sta-
tistical evidence (2-sided p-value < 0.001) that the percent
linoleic acid content changed during storage. After 3 mo frozen
storage a small decrease was observed in relative percent of
linoleic acid, which tended to stabilize later (Table 2). This sug-
gested possible autoxidation of the fatty acid due to a self-cat-
alytic free radical mechanism.

There was significant evidence that the mean percent linolenic
acid content varied during frozen storage (2-sided p-value <
0.0001). The relative percent linolenic acid content in the whole
kernels decreased during frozen storage (Table 2). Regression
analysis indicated that the mean % linolenic acid content was
dependent on blanching treatment (2-sided p-value < 0.0001).
Unblanched samples decreased at a faster rate than blanched
samples in both cultivars, suggesting enzymatic oxidation of lin-
olenic acid during storage. Poca et al. (1990) characterized a
corn lipoxygenase isozyme (L1) with high affinity for α-lino-
lenic acid leading to the formation of 13-hydroperoxides. In ad-
dition, autoxidation by free radical mechanisms was suggested
by the decrease in relative percent linolenic acid of blanched
samples during frozen storage, especially at 15 min where both
enzymes were inactivated.

Peroxide value

The peroxide value was related to cultivar (2-sided p-value
< 0.01) and blanching treatment (2-sided p-value < 0.01).
Changes in peroxide value during storage were different in the
two cultivars (2-sided p-value < 0.02). The peroxide value of
unblanched Jubilee increased (Fig. 1) an average of 50% from
the sixth to the ninth month of storage. The Jubilee, 9 min
blanched, sample showed some increase in peroxide value but
no increases were observed in the Jubilee, 15 min blanched,
sample nor any GH 2684 samples.

The highest peroxide value obtained was 0.5 meq O_2/kg lipid
in the unblanched Jubilee sample after 9 mo storage. The per-
oxide value is a good guide for quality of a lipid; freshly refined
fats should have peroxide values < 1 meq O_2/kg (Rossell,
1989). All peroxide values we found were much less than that.

Packaging materials showed no effect on control of polyun-
saturated fatty acid degradation. The low rates of lipid degra-
dation in all samples during 9 mo frozen storage suggested that
degradation was not related to oxygen concentration around the
corn.

Moisture

The initial moisture content in the sweet corn was, on aver-
age, 72.5%. After 9 mo frozen storage, the different packaging
materials used were related to the control of moisture loss from
corn kernels (2-sided p-value < 0.01)(Fig. 2). Ears stored in
Cryovac B and E bags showed the best moisture retention
(72.2% final moisture), followed by polyethylene bags (71.4%).
Those stored without packaging materials showed severe de-
hydration (70.1%).

SUMMARY

PACKAGING MATERIALS had no effect on controlling PUFA deg-
radation or enzymatic activity; however, they had an important
effect on preventing moisture loss. Blanching of ears of corn
reduced LOX and POD activities but had little effect on PUFA
degradation during 9 mo storage at −20°C.

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Ms received 11/7/94; revised 3/2/95; accepted 3/30/95.

Nitrogen-to-Protein Conversion Factors for Some Common Edible Mushrooms

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——————— ABSTRACT ———————

Mushrooms (13) common to Japan were analyzed for total nitrogen (TN), amino acid nitrogen (AAN), amide-N and nonprotein nitrogen. The distribution of protein nitrogen averaged 67% to TN. Averaged nitrogen-to-protein conversion (N:P) factor was 5.99. Application of the factor for estimation of protein content resulted in a considerable difference from amino acid residue (AAres) levels, due to the high level of nonprotein nitrogen. A net N:P factor, defined as the proportion of AAN to TN, was proposed. The averaged value of 3.99 was derived from analytical TN and AAN data of mushrooms. Calculated protein values using this factor were in good agreement with AAres.

Key Words: mushrooms, protein, nitrogen, amides, nonprotein nitrogen

INTRODUCTION

THE AMOUNT OF TOTAL PROTEIN in foods is usually calculated by multiplying its Kjeldahl nitrogen content by the nitrogen to protein conversion factor (N:P factor). Although special factors for conversion of nitrogen to protein are used for some foods (Jones, 1941), the value of 6.25 is applied for most plant and animal proteins, assumed to contain 16% nitrogen. However, this assumption is invalid for foods that contain other nitrogenous compounds such as nucleic acids, ammonium and nitrogenous glycosides. Use of the common factor 6.25 for such foods may result in overestimation of protein content. Therefore, a more practical N:P factor, designed to allow for the effects of nonprotein nitrogen is needed.

Fruiting bodies of mushrooms contain notable nonprotein nitrogen compounds, such as nucleic acids and chitin. The former, responsible for the umami taste of mushrooms, contain ≈15–16% nitrogen (Benedict, 1987). Chitin, a polymer of N-acetyl-glucosamine, exists in the cell wall of mushrooms. The nucleic acid nitrogen and chitin nitrogen contents should affect the N:P factor for mushrooms. Mushrooms were selected as model foods to explore the method of determining N:P factors. Although amino acid compositions and N:P factors of common cultivated mushrooms have been reported by Ogawa et al. (1987), amide-N were not estimated. Furthermore, they made reference to only glucosamine as nonprotein nitrogen.

Our objectives were to determine a more accurate N:P factor for mushrooms based on amino acid composition including amide-N and the distribution of nitrogen in protein or other nitrogenous components, and to propose a reliable procedure for routinely converting total nitrogen data to protein content.

MATERIALS & METHODS

Materials

Freshly harvested fruiting bodies of cultivated mushrooms, *Flammulina velutipes* (Enokitake), *Grifola frondosa* (Maitake), *Lentinus edodes* (Shiitake), *Pholiota nameko* (Nameko), *Pleurotus ostreatus* (Hiratake) and *Tricholoma giganteum* (Nioushimeji) were kindly supplied by some producers. *Armillariella mellea* (Naratake), *Hygrophorus russula* (Sakurashimeji), *Lyophyllum shimeji* (Honshimeji), *Panellus serotinus* (Mu-

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kitake), *Ramaria botrytis* (Houkitake), *Sarcodon aspratus* (Koutake) and *Suillus grevillei* (Hanaiguchi) were collected. The fresh fruiting bodies were washed with distilled water, then freeze-dried. The freeze-dried samples were ground in a mill to pass through a 0.3mm sieve.

Analyses

Amino acids composition. Amino acids analyses of each mushroom were carried out using an amino acid analyzer (HITACHI 835) after 6N HCl hydrolysis at 110°C for 24 hr under vacuum. Cysteine and cystine were measured as cysteic acids (reported as Cys/2), and methionine was measured as methionine sulfone after perfomic acid oxidation and 6N HCl hydrolysis at 150°C for 20 hr according to the method of Moore (1963). The tryptophan analysis was done on a $Ba(OH)_2$ hydrolysate as described by Tkachuk and Irvine (1969). The amide-N content was determined by titration of the NH_3 liberated by 2N HCl hydrolysis of the sample protein (Bailey, 1937) using a Conway vessel. As a blank, NH_3 in the 70% ethanol extract of the samples was also measured (designated NH_3-N). The moles of amide-N were assigned on a proportional basis to the moles of aspartic acid and glutamic acid in the samples to determine the asparagine and glutamine values (Sosulski and Imafidon, 1990). Free amino acids were determined by automatic amino acid analysis according to a method described previously (Sato et al., 1985).

Chitin nitrogen determination. Chitin fraction was isolated as insoluble residue after alkali-acid treatment according to the method employed by Yanase (1975) and quantitatively estimated by Boas's method (1953). The method of isolation (Yanase, 1975) is based on the glucosamine liberation from chitin fraction by 6N HCl hydrolysis at 114°C for 6 hr. Nitrogen content in chitin (Chitin-N) was calculated from the amount of glucosamine.

Nucleic acid nitrogen determination. Four purine and three pyrimidine bases in mushrooms were quantitatively analyzed using HPLC. The samples (100 mg) were extracted with 25 mL of 5% perchloric acid (PCA) under reflux at 100°C for 20 min. The extract was concentrated and subsequently hydrolyzed by concentrated PCA at 100°C for 1 hr (Marshak and Vogel, 1951). The hydrolysate was neutralized with 33% potassium hydroxide and applied to the cation exchange resin column (Yanaco SCX-1001. 5×230 mm). Elution of bases from the column was made with 0.05M dibasic ammonium phosphate, containing 2% ethanol at 45°C (flow rate 0.5mL/min.; sample size 0.1mL). The effluent was continuously monitored for ultraviolet absorption at 254nm (Shinoda et al., 1981). Each purine and pyrimidine base content was evaluated from the proportion of the peak area to that of standard material. Nucleic acid nitrogen (NAN) of each mushroom was calculated by multiplying concentration of each base by the theoretical percentage nitrogen.

RESULTS & DISCUSSION

TOTAL AMINO ACIDS (Total AA), free amino acids (Free AA), contents of asparagine and glutamine (Asn+Gln), amino acid residue (AAres) and total nitrogen contents in amino acids (AAN) in mushrooms were compared (Table 1). In addition, N:P factor of each mushroom based on quantitative amino acid composition data. including estimation of the amide-N were also compared. Total AA value is the sum of the weight of each amino acid, including Asn and Gln calculated from Amide-N and part of Asp and Glu. On average, the sum of Asn and Gln was 1.71 ± 0.80 mg (mean $\pm$ SD)/100g of dry matter. The average value of free AA was 3.40 ± 2.03g/100g of dry matter. The AAres value, defined as total AA minus the element of water, represents a true weight of protein in the sample. These values ranged from 12.02g in *A. mellea* to 28.33g in *P. ostreatus*/100g of dry matter. The AAN value is the corresponding

Table 1—Calculation of N:P factor of common edible mushrooms

	Total AA[a]	Free AA	Asn+Gln[b]	AAres[c]	AAN[d]	N:P factor
		(g/100g of fruiting bodies, dry basis)				(AAres/AAN)
Cultivated						
F. velutipes*	20.94	7.26	1.81	17.94	3.00	5.98
G. frondosa*	21.20	1.27	1.61	18.15	3.00	6.05
L. edodes**	21.72	4.71	2.02	18.62	3.30	5.64
P nameko*	20.75	2.89	1.38	17.74	2.88	6.16
P. ostreatus**	32.95	7.83	3.97	28.33	4.80	5.90
T. giganteum	15.44	2.94	1.15	13.23	2.21	5.99
Native						
A. mellea	14.10	0.93	0.74	12.02	1.94	6.20
H. russula	20.56	2.42	2.30	17.53	2.92	6.00
L. shimeji	19.71	4.35	1.84	16.76	2.77	6.05
P. serotinus	15.71	2.11	0.58	13.43	2.18	6.16
R. botrytis	17.25	2.09	1.37	14.71	2.58	5.70
S. aspratus	23.21	2.96	1.75	19.87	3.28	6.06
S. grevillei	20.76	2.45	1.67	17.76	3.00	5.92
avg	20.33	3.40	1.71	17.39	2.91	5.99
S.D.	± 4.515	± 2.032	± 0.800	± 3.902	± 0.678	± 0.159

[a] Total amino acids including free amino acids and Asn+Gln.
[b] Calculated from amide-N.
[c] Total AA minus the element of water.
[d] Nitrogen content in Total AA.
* mean of two samples
** mean of three samples

Table 2—Distribution of N components in mushrooms and net N:P factors

	Total N	AAN[a]	Chitin-N[b]	NAN[c]	NH3-N[d]	Total	net N:P factor
	(% of DM)		(mg/g of Total N)				(AAres[e]/Total N)
Cultivated							
F. velutipes*	3.94	759.89	92.18	77.00	15.83	944.90	4.55
G. frondosa*	4.19	716.20	63.39	81.04	15.90	876.53	4.33
L. edodes**	4.11	800.84	95.71	75.19	14.49	986.52	4.53
P. nameko*	4.00	715.66	78.29	92.85	22.49	909.29	4.44
P. ostreatus**	6.82	706.23	51.63	79.93	6.04	843.83	4.15
T. giganteum	3.99	552.63	80.53	43.99	27.05	704.20	3.32
Native							
A. mellea	2.83	687.85	162.75	65.19	34.23	950.02	4.25
H. russula	4.89	596.95	82.71	26.86	12.12	718.64	3.58
L. shimeji	7.04	393.82	56.75	41.43	9.54	501.54	2.38
P. serotinus	4.99	436.38	44.81	43.27	21.32	545.78	2.69
R. botrytis	3.71	696.80	137.90	52.61	13.92	901.23	3.96
S. aspratus	3.87	847.94	74.74	61.01	27.19	1010.88	5.13
S. grevillei	3.95	758.32	96.20	83.77	13.46	951.75	4.50
avg	4.49	666.89	85.97	63.40	17.99	834.24	3.99
S.D.	± 1.157	± 130.405	± 31.976	± 19.446	± 7.768	± 159.331	± 0.758

[a] Nitrogen content in Total AA.
[b] Nitrogen content in chitin.
[c] Nucleic acid nitrogen.
[d] NH3 nitrogen.
[e] see Table 1.
* mean of two samples
** mean of three samples

weight of nitrogen in these AAres. The value of AAN ranged from 1.94g in A. mellea to 4.80g in P. ostreatus/100g dry matter. Protein content, that is, AAres value, as defined, includes all free and bound amino acids as forming part of the total protein content. Therefore, the elements of water were subtracted from not only the bound amino acids but also from free amino acids. The N:P factor was then determined from the ratio of AAres to AAN in each mushroom. Resulting values varied from 5.64 for L. edodes to 6.20 for A. mellea. The average N:P factor for 13 mushrooms was 5.99 ± 0.16 (mean ± SD). This average differed slightly from 5.61 reported by Sosulski and Imafidon (1990).

By use of Chitin-N, NAN and NH3-N data we obtained, it was possible to structure a general table of nitrogen components in mushrooms (Table 2). Total nitrogen (total N) values determined by Kjeldahl method of each mushroom were also included. AAN occurring in either free or bound forms accounted for 393.82 mg in L. shimeji to 847.94 mg in S. aspratus (666.89 mg on the average)/g total N. Recovery of only 67% of the total N in the form of AAN indicates that 33% of the total N may be due to the presence of other nitrogenous sources.

Chitin-N ranged between 44.81 mg in P. serotinus and 162.75 mg in A. mellea/g of total N. The value of NAN ranged from 41.43 mg in L. shimeji to 92.85 mg in P. nameko/g of total N. The average level of NH3-N was 17.99 mg/g total N. On average, the sum of these nitrogen contents was 834.24 ± 159.33 mg (mean ± SD)/g of total N. Variability exists among species, in particular the values of L. shimeji and P. serotinus were very low (501.54 mg and 545.78 mg/g total N, respectively). These figures might reflect the existence of other nitrogenous compounds in these mushrooms, but further investigations were not made.

The averaged N:P factor for mushrooms calculated from AAres and AAN was 5.99. However, application of the factor would result in overestimation of protein owing to the presence of high nonprotein nitrogen (NPN) contents. As mentioned above, NPN in mushrooms accounted for 33% of the total N. Since the Kjeldahl method cannot distinguish between NPN and protein the NPN should be excluded from total N to determine protein content more accurately by using the N:P factor. It is very difficult to analyze various kinds of NPN of all samples. A net N:P factor was proposed for conversion of total N to protein content by consideration of NPN. That is, the net N:P factor was defined as the ratio of AAres to Total N. As shown (Table 2) the net N:P factors of mushrooms ranged from 2.38 (L. shimeji) to 5.13 (S. aspratus), average 3.99 ± 0.76.

Table 3—Comparison of calculated protein contents (g/100g of fruit ng bodies, dry basis)

| | AAres | Calculated protein content | | | |
		Total N X N:P factor[a]	(Protein N[b]) X N:P factor[a]	Total N X 3.99[c]	Total N X 6.25
Cultivated					
F. velutipes*	17.9	23.6	19.2	15.7	24.6
G. frondosa*	18.2	25.3	21.3	16.7	26.2
L. edodes**	18.6	23.2	18.9	16.4	25.7
P. nameko*	17.7	24.6	19.9	16.0	25.0
P. ostreatus**	28.3	40.2	34.7	27.2	42.6
T. giganteum	13.2	23.9	20.3	15.9	24.9
Native					
A. mellea	12.0	17.5	12.9	11.3	17.7
H. russula	17.5	29.3	25.8	19.5	30.6
L. shimeji	16.8	42.6	38.0	28.1	44.0
P. serotinus	13.4	30.7	27.4	19.9	31.2
R. botrytis	14.7	21.1	16.8	14.8	23.2
S. aspratus	19.9	23.5	19.6	15.4	24.2
S. grevillei	17.8	23.4	18.9	15.8	24.7
avg	17.4	26.9	22.0	17.9	28.0
S.D.	± 3.90	± 6.97	± 6.24	± 4.62	± 7.23

[a] The specific N:P factor (see Table 1).
[b] Total N minus nonprotein nitrogen (chitin-N + NAN + NH_3-N).
[c] Averaged net N:P factor (see Table 2).
* mean of 2 samples
** mean of 3 samples

Five methods to calculate protein content of mushrooms were compared (Table 3).

(1) summation of AAres content of each mushroom.

(2) multiplication of total N by the N:P factor specific for each mushroom (see Table 1).

(3) multiplication of residual N after subtracting NPN from total N, by specific N:P factor.

(4) multiplication of total N by net N:P factor (3.99).

(5) multiplication of total N by 6.25, commonly applied factor for foods.

The best estimation of protein content was the summation of AAres. When these calculated values were compared, considerable differences were found between AAres and the protein content calculated by the traditional factor, 6.25. Similar differences were observed between AAres and results by total N × the specific N:P factor for each mushroom. However, good agreement was found between AAres value and the protein content calculated by total N minus NPN × the specific N:P factor, except for a few mushrooms. Also, protein contents calculated by using the net N:P factor were in good agreement with AAres.

These results indicated that the net N:P factor was a reliable and practical conversion factor for estimation of protein contents of mushrooms. Furthermore, this method would be applicable to many other foods. In particular, for foods that contain a high level of NPN, a net N:P factor could give a more accurate protein value.

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Ms received 9/12/94; revised 3/20/95; accepted 4/8/95.

Stability of Spray-Dried Encapsulated Carrot Carotenes

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ABSTRACT

Encapsulation with various dextrose equivalent (DE) hydrolyzed starches affected stability of α- and β-carotene in spray-dried carrot powders. Degradation of α- and β-carotene during storage of the powders at temperatures ranging from 37 to 65°C followed first-order kinetics and both degraded at the same rate. Hydrolyzed starch of 36.5DE was superior to 4, 15, and 25DE in improving retention of the carotenes. Carotenes encapsulated with 36.5DE hydrolyzed starch had a predicted half-life of 450 days at 21°C compared to 2 days for carrot juice spray dried alone. Increasing the proportion of carrier decreased the carotene degradation rate and similarly decreased surface carotene. Air was critical in carotene stability, but exposure of encapsulated carrot powders to light did not accelerate degradation.

Key Words: carrots, beta-carotene, starch, thermal degradation, encapsulation

INTRODUCTION

CAROTENOIDS are responsible for many natural yellow to red coloring hues in foods. Epidemiological studies of fruits and vegetables with above average carotenoids have been associated with 30–50% lower incidence of several types of cancer including, lung, stomach and skin (Mayne et al., 1994; Menkes et al., 1986; Zeigler, 1989). Murakoshi et al. (1992) reported that α-carotene, but not β-carotene, reduced lung tumors, and also decreased spontaneous liver carcinogenesis and suppressed skin carcinogenesis. Carrots are one source of carotenoids that contain a high level of α-carotene (Bureau and Bushway, 1986). However, carrots usually are not dried as a source of carotene because drying frees oxidative enzymes, exposes the carotenes to heat and lowers water activity. This leads to losses in vitamin A activity, carotene degradation, color deterioration and potential off-flavor due to production of oxidation products. Such oxidation products have little or no pigmentation, provitamin A (Quakenbush and Smallidge, 1986) or oxygen quenching activity. Many studies (Baloch et al, 1977 a-c; MacKinney et al., 1958; DellaMonica and McDowell, 1965; Schadle et al., 1983) on losses of carotenoids in dehydrated carrots have been done prior to development of analytical techniques (such as high performance liquid chromatography, HPLC), that enable the stability of α- and β-carotene to be assessed independently.

An increasing trend in food and pharmaceutical industries is toward replacing synthetic additives with natural products. However, creating suitable water dispersible forms of carotenoids is difficult because of the limited solubility of pure carotene crystals. Encapsulation is a potential approach to transform liquids into stable and free flowing powders which are easy to handle and incorporate into dry food systems. Among encapsulation methods, spray drying is one of the best known and most economical. Hydrolyzed starches have the advantage of being very cost effective encapsulating agents which have been shown to protect against oxidative deterioration of orange oil (Anandaraman and Reineccius, 1986). Beatus et al. (1985) spray-dried paprika oleoresin encapsulated with 15DE maltodextrin, gum arabic, gelatin and sodium caseinate and found the maltodextrin was the best agent to protect against oxidation of carotenoids

during storage. However, no investigations have been reported on the relation of dextrose equivalent (DE) to stability of spray dried encapsulated carotenoids.

Our objectives were to evaluate hydrolyzed starches of various DEs as encapsulating agents for spray drying of carrot juice and to determine the effects of light exposure, temperature, and carrier level on the stability of spray dried carrot carotenes.

METHODS & MATERIALS

Materials

HPLC grade methanol and OmniSolv® grade water, acetonitrile and hexane were obtained from EM Science (Gibbstown, NJ). Certified grade stabilized tetrahydrofuran (THF) containing 250 ppm butylated hydroxytoluene (BHT) was purchased from Fischer Scientific (Fair Lawn, NJ). Hydrolyzed starch samples (4DE, 15DE, 25DE, and 36.5DE) were obtained from Grain Processing Corporation (Muscatine, IA) and concentrated carrot coagulum was obtained from Humanetics Corp. (Chaska, MN). The carrot coagulum was processed under the following procedure prior to receiving. Imperator-type carrots (Daucus carota) were cleaned. sanitized with 200 ppm chlorine, rinsed, disintegrated with a Comitrol disintegrator (Urschel Laboratories Inc., Model 3610, Valparaiso, IN) and pressed in a Bepex S type screw press (Bepex Corp., Minneapolis, MN). CaCl$_2$ was added as a 15% slurry to the concentrated carrot juice to obtain a final CaCl$_2$ concentration of 0.1% on a w/w basis. The mixture was then pasteurized at 80°C for 60 sec with a scraped surface heat exchanger (APV, Lake Mills, WI), cooled to 50°C, concentrated to 13.5% total solids by ultrafiltration (Komline-Sanderson Membrane Systems cross flow membrane, Pea Pack, NJ). The concentrated carrot coagulum samples (ca 300g) were quiescently frozen in polyethylene bags at −20°C until further experiments.

Encapsulation of carrot coagulum

The frozen carrot coagulum was allowed to thaw at refrigerated conditions (ca. 4°C) for 2 to 3 days and homogenized with a high shear lab homogenizer (Gifford-Wood Model 11.81, Greerco Corp., Hudson, NH) (ca. 3 min). Dry hydrolyzed starch (4DE, 15DE, 25DE or 36.5DE) was combined with the carrot coagulum and homogenized. The feed composition was 3.97 kg carrot coagulum and 1.03 kg dry hydrolyzed starch except in the experiment where carrier level was varied. For comparing the four hydrolyzed starches of various DEs, the feed composition was 20% dry starch to carrot coagulum on a wet weight basis. The dispersion was then heated in a water bath (38°C) to dissolve the hydrolyzed starch, followed by high shear mixing for 5 min to ensure feed homogeneity prior to spray drying. This solution was fed to a Niro Utility Model spray drier (Niro Atomizers, Ltd., Copenhagen, Denmark) equipped with a centrifugal wheel atomizer. The spray drier was operated at an inlet temperature of 200 ± 5°C and 100 ± 5°C outlet temperature. Carrot coagulum alone was spray-dried as a control. The powders were allowed to cool to room temperature, stored in covered glass jars wrapped with aluminum foil to exclude light and kept at −29°C prior to analysis.

Extraction of carotenoids from encapsulated carrot powder

Spray dried powder (0.2 g) was accurately weighed into a 50-mL centrifuge tube, reconstituted by the addition of 3 mL of water and vortexed. To extract the carotenes, methanol (5 mL) was added to the slurry, vortexed and kept for 3 min at room temperature followed by addition of 10 mL of stabilized THF. The mixture was centrifuged at 2200 × g (Beckman Instruments, Inc., Model J2-21, Palo Alto, CA) for 5 min at 4°C to remove precipitated encapsulating material and carrot coagulum. The extract was removed by decantation. A second extraction was done on the residue using 5 mL of methanol. The sample was centrifuged as described. A third extraction with 5 mL THF followed by centrifugation

was performed as described when needed, to produce a gray/white precipitate without any remaining orange color. The pooled extracts were filtered (0.45 μ) and analyzed by HPLC. Samples were stored in amber vials at 4–6°C for a maximum of 5 hr until analysis. Two replicate extractions of each spray dried sample were analyzed. Initial α- and β-carotene was determined prior to storage. No loss was observed in the α- and β-carotene content of the frozen samples over the duration of the study.

Standard solutions

all-trans-α-Carotene and *all-trans*-β-carotene were purchased from Sigma Chemical Co. (St. Louis, MO). Stock solutions were prepared in hexane monthly and stored in the dark at −20°C. Concentrations of standards were spectrophotometrically determined daily by measuring the absorbance at 453 nm (E$^{1\%}$ = 2592) for β-carotene and 446 nm (E$^{1\%}$ = 2725) for α-carotene (De Ritter and Purcell, 1981; National Research Council, 1972) Two concentrations of each carotene standard in the range 0.73–3.9 g/mL were injected daily for quantification by peak area to determine carotene content. Standards of 9-*cis*-, 13-*cis*- and 15-*cis*-β-carotene were obtained from Hoffmann-La Roche, Inc. (Basel, Switzerland) and prepared in hexane. Sample carotenes were identified by comparing peak retention times and spectral scans with standards.

Chromatographic procedure

HPLC analysis of carotenoids was carried out with a Vydac 201TP54 column (C18, 5 μ particle size, 4.6 mm i.d. × 25 cm, Vydac, Hesperia, CA). A mobile phase of methanol: acetonitrile: water (88:9:3 v/v/v) was used at a flow rate of 1 mL/min maintained by a Waters pump model M-6000A (Waters Associates, Inc., Milford, MA). Prior to HPLC analysis, mobile phase was degassed by sonication and filtered through a 0.45 μ nylon filter. The mobile phase and column were similar to those reported by Bryant et al. (1992). A model 7125 Rheodyne fixed-volume loop injector with a 10-μL loop was used with fixed wavelength detection or a 50-μL loop was used for diode array analysis. The column temperature and mobile phase were maintained at 38°C with a column water jacket (Alltech, Deerfield, IL) and controlled temperature circulator (Fischer Scientific, model 950, Pittsburgh, PA).

Two detection systems were used to monitor column eluant at 436 nm. System I used a Spectroflow 757 Kratos analytical absorbance detector (Ramsey, NJ) and a Waters QA-1 integrator (Marlborough, MA). System II was a Hewlett Packard 1040A diode array detector controlled by a Hewlett Packard Chem Station (St. Paul, MN) and determined the spectrum of the eluant (250–600 nm). Both systems allowed baseline separation of α- and β-carotene and could separate 9-*cis*-, 13-*cis*-, and 15-*cis*-isomers of β-carotene from its *all-trans* form.

Moisture determination-Karl Fischer titration

Duplicate samples (6g) of powders encapsulated with 20% 4DE, 15DE, 25DE or 36.5DE hydrolyzed starch were accurately weighed into Erlenmeyer flasks and covered with 50 mL anhydrous methanol. The flasks were stoppered and agitated on an incubator shaker (New Brunswick Scientific, model G24, New Brunswick, NJ) at 200 rpm for 16 hr at 4°C to extract moisture. The methanol extracts (100 mL) were injected into an automatic Karl Fischer titration apparatus (automatic moisture analyzer, Aquatest-IV, Photovolt Corporation, New York, NY). A blank was determined simultaneously to account for any moisture in the anhydrous methanol.

Water activity determination

Water activity of the spray-dried samples encapsulated with 20% 4DE, 15DE, 25DE or 36.5DE hydrolyzed starch was determined by an AquaLab water activity analyzer (Decagon Devices, Inc., Model CX-2, Pullman, WA). Samples were analyzed in duplicate.

Storage stability tests

Effect of light. Spray-dried carrot powders encapsulated with 4DE and 36.5DE hydrolyzed starch were used to determine effects of light exposure. Samples of 1.75 g were put in 55 × 10 mm Petri dishes and placed in a 50 × 25 × 30-cm black coated light box constantly exposed to 2150 lux of light. Constant light intensity was provided by three standard 15 watt. cool-white, fluorescent lights (General Electric) ≈25 cm from the samples. Samples were placed 5 cm from the bottom of the

box on a translucent plate. Unexposed samples were placed in plastic Petri dishes protected with aluminum foil and placed in the light box. The box was placed in an incubator room and maintained at 45°C inside the box. Duplicate unexposed and exposed samples were taken weekly for HPLC analysis. Degradation of α- and β-carotene was followed over 8 wks storage.

Effect of hydrolyzed starch DE and storage temperature. Samples (≈0.75g each) of spray-dried carrot coagulum powder with 4DE, 15DE, 25DE or 36.5DE starch as an encapsulating agent were hermetically-sealed in 202 × 214 steel tinplate cans to maintain constant moisture without any attempt to remove oxygen. Eight canned samples were then placed in controlled temperature rooms (37°C and 45°C) or in an incubation oven (65°C). Duplicate samples were removed from 37°C incubation bi-weekly, 45°C weekly and 65°C daily at eight intervals. HPLC analyses for carotene were conducted at each interval and degradation occurred past 70% loss by the eighth sampling. Controls of spray dried carrot coagulum alone without encapsulating agent were incubated in cans at 21°C and analyzed daily because of rapid degradation.

Effects of atmosphere. Samples (1.0 g) of dried powders prepared with hydrolyzed starch of each DE were weighed into plastic Petri plates (55 × 10 mm) and placed in desiccators containing saturated LiCl solution (a$_w$ = 0.112) protected from light with aluminum foil. For aerobic conditions, seven samples of each DE treatment were stored in a desiccator closed in air. Duplicate samples were stored under an anaerobic hood in a desiccator flushed with a mixture of 5% carbon dioxide, 10% hydrogen and 85% nitrogen. The anaerobic hood was constructed such that only the sample being removed was exposed to air. Duplicate spray-dried samples were analyzed weekly from anaerobic and aerobic conditions for carotene degradation over 7 wk storage.

Effects of carrier level. Samples were spray-dried with varied in-feed composition. Powders were prepared by combining carrot coagulum with 10, 15, 20 or 25% 15DE dry hydrolyzed starch on a wet basis resulting in in-feed total solids levels of 22, 26, 31 and 39% respectively in 5-kg batches. Samples (eight, 1 g each) of each carrier level were hermetically-sealed in 202 × 214 steel tinplate cans without any attempts to remove oxygen and placed in a 50°C incubator oven. HPLC analysis of carotenes was conducted on duplicate powders at 8 intervals over 9 days incubation.

Surface carotene

To determine the amount of surface carotene on encapsulated powders, duplicate samples (0.1 g) of the carrot powders were accurately weighed into 50-mL centrifuge tubes and extracted with 10 mL hexane. The mixture was centrifuged at 2200 × g for 5 min at 4°C, decanted and the precipitate extracted with additional 10 mL hexane. Extracts were combined and analyzed for α- and β-carotene by HPLC to determine the fraction of carotene not encapsulated but retained on powder surfaces. Similar procedures have been used for surface oil determination in flavor encapsulation analysis using hexane as the surface extracting solvent (Subramaniam, 1984).

Data analysis

The reaction order, rate constants, activation energies and 95% confidence limits for the degradation rates of α- and β-carotene were determined with the aid of the Macintosh program *Water Analyzer Series— Reaction Kinetics Program Version 2.09* (Labuza et al., 1991). This program is based on equations from kinetic rate determinations using the integral method (Labuza, 1984). Linear regression on the data over time is applied for a large change in concentration and the 95% confidence limits of the slope are calculated by the equation:

$$95\% \text{ CI} = x \pm t \frac{\sigma}{\sqrt{n_t}}$$

where: x = mean of samples, σ = sample standard error, n$_t$ = number of data points, and t = Student distribution for the number of degrees of freedom (n-2). Statistical differences between rate constants were determined by the method of Labuza (1984).

RESULTS & DISCUSSION

Storage stability evaluation

Stabilities of major carrot carotenes in spray-dried encapsulated powders were evaluated under various conditions of light, temperature and atmosphere. The kinetics of degradation of α-

and β-carotene were monitored over the incubation period and reaction orders, rate constants, and half-life values of reactions were determined. Based on r^2 as the criterion for reaction order we determined that a first-order reaction (over zero, second, or third order) best described the degradation of both α- and β-carotene under these conditions. Previous researchers investigating carotene degradation kinetics have also concluded that degradation due to heat, oxidation and light followed first-order or pseudo-first-order behavior. Pesek and Warthesen (1987) investigated carotene degradation in a vegetable juice system and found α- and β-carotene followed first-order degradation kinetics. A similar conclusion was also arrived by Chou and Breene (1972) and Stevanovich and Karel (1982) in β-carotene degradations in model dry systems.

Although HPLC analysis could separate cis isomers, the change from all-trans to cis-trans isomers was not observed during processing or storage of powders. Cis isomers of α- and β-carotene elute within 2 min of the *all-trans* forms. No detectable *cis* isomer peaks were found (Fig. 1, 2) in the degraded samples. Degradation appeared to involve direct oxidation rather than isomerization of the carotenes. Farhangi and Valadon (1981) also observed degradation of carotenoids without isomerization during acid processing and storage of mung bean sprouts.

All hydrolyzed starch encapsulated carrot products prepared with 20% carrier retained about their initial (>90%) α- and β-carotene when stored under anaerobic conditions at 37°C for 7 wk. This suggested that the mechanism of degradation was likely direct oxidation of the carotenes without destabilization through isomerization. Oxygen was a critical factor in β-carotene degradation (Chou and Breene, 1972; Goldman et al., 1983). Simpson (1985) also found oxidation was the major cause of β-carotene destruction. Exclusion of oxygen during storage of powders would extend their shelf-life.

Effect of light

To evaluate the effects of light on α- and β-carotene stability in encapsulated carrot powders, 4DE and 36.5DE hydrolyzed starch encapsulated carrot powders were selected for storage studies. Typical chromatograms of the extract of 4DE and 36.5DE encapsulated carrot powder initially and after 56 days of light exposure were compared (Fig. 1 and 2). The chromatograms showed the presence of typical α- and β-carotene found in carrots. Initial α-carotene content was 605 and 611 g/g for the 4DE and 36.5DE encapsulated product, respectively, and 1514 and 1580 g/g for β-carotene. The ratio of α-carotene to β-carotene agreed with that reported previously by Bushway (1986) and Tee and Lim (1991) for raw carrots.

The HPLC chromatographic conditions used for the separation were similar to those reported by Bryant et al. (1992). However, conditions were slightly modified to control column temperature at 38°C to improve reproducibility and separation of all-trans-α-carotene from all-trans-β-carotene and the possible 9-cis-, 13-cis-, and 15-cis-β-carotene.

The retention of the carotenes, in either powder was about the same under light and dark conditions. In general, autooxidation of carotene is catalyzed by a variety of factors such as light, metal ions, and photosensitizers. However, after 8 wk incubation of the carrot powders in either light or dark conditions at 45°C, total degradation was 90% for both α- and β-carotene in the 4DE encapsulating powder compared to 70% loss of both α- and β-carotene in the 36.5DE powder (Fig. 3 and 4). This indicated that exposure to light did not accelerate degradation of the carotenes. Kearsley and Rodriguez (1981) found conversely that light accelerated degradation when investigating β-carotene dispersed in water. They reported that after light exposure for 144 hrs at 25°C, 75% of the β-carotene was destroyed compared to 33% loss in dark conditions. Pesek and Warthesen (1987) also reported that degradation of α- and β-carotene in a vegetable juice system was accelerated by exposure to light at 4°C.
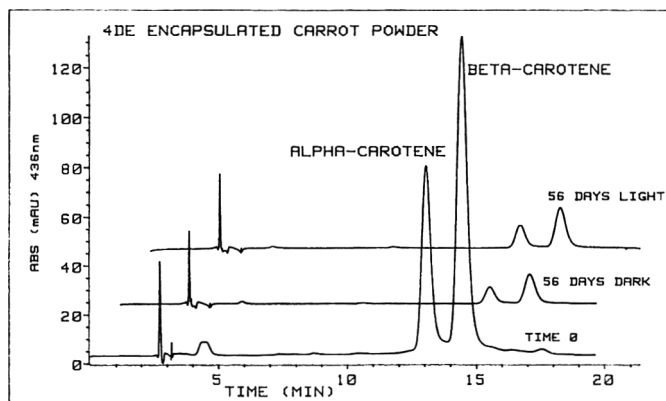

Fig. 1—Representative chromatograms of the extract of spray-dried carrot powders encapsulated with 4DE hydrolyzed starch initially and after storage at 45°C unexposed or exposed to 2150 lux of light.
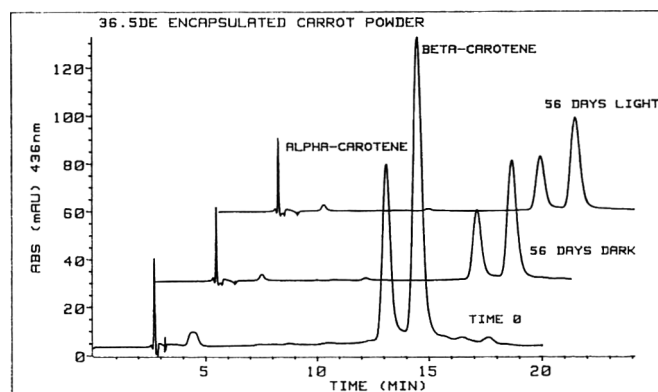

Fig. 2—Representative chromatograms of the extract of spray-dried carrot powders encapsulated with 36.5DE hydrolyzed starch initially and after storage at 45°C unexposed or exposed to 2150 lux of light.

We hypothesize that degradation of α- and β-carotene occurs without *trans-cis* isomerization by the mechanism of direct oxidation in either light or dark conditions. The resulting oxidation products have no coloring properties and thus do not absorb at 436 nm. Possibly under the dry conditions evaluated, trans-cis isomerization of the carotenes was physically inhibited and may require solubility for molecular movement. Light may not have accelerated degradation of the carotene due to lack of mobilized metal catalysts.

The degradation rates and half-life values of the major carrot carotenes were compared (Fig. 5) at 45°C in unexposed and exposed carrot powders prepared with 20% (wb) 4DE or 36.5DE hydrolyzed starch. Degradation of α- and β-carotene occurred at the same rate (p < 0.05). This is the first report that has compared α- to β-carotene degradation in a dried carrot system. Pesek and Warthesen (1987) found that α- and β-carotene degraded at the same rate in a vegetable juice system also there were 2× greater α- and β-carotene degradation rates for the 36.5DE compared with 4DE encapsulated carrot powder, indicating 36.5DE hydrolyzed starch was a preferable encapsulating agent.

Effect of hydrolyzed starch DE and storage temperature

An increase in storage temperature led to an increase in rate constant (k) for both α- and β-carotene in all four hydrolyzed starch encapsulated carrot products. For the four powders with varying DEs of hydrolyzed starch as encapsulating agents, rate constants at 21°C were predicted with use of Arrhenius equa-
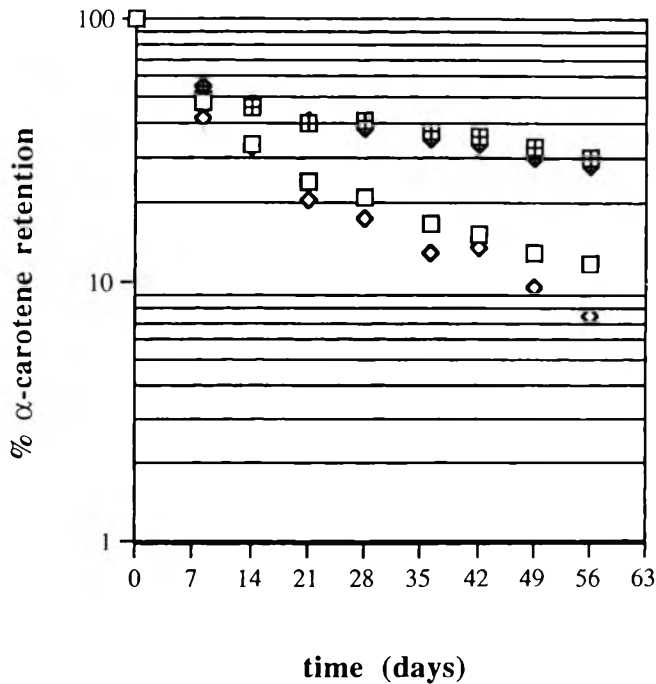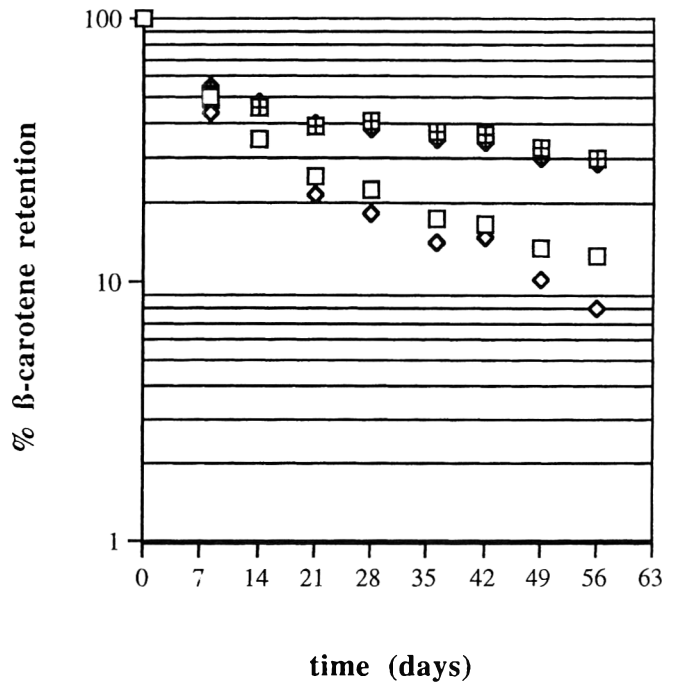
Fig. 3—First-order degradation plots for α-carotene in spray-dried carrot powders encapsulated with 4DE or 36.5DE hydrolyzed starch unexposed or exposed to 2150 lux of light. Each point represents an average of duplicate analyses. □ 4DE exposed, ◊ 4DE unexposed, ⊞ 36.5DE exposed, ◆ 36.5DE unexposed.

Fig. 4—First-order degradation plots for β-carotene in spray-dried carrot powder encapsulated with 4DE or 36.5DE hydrolyzed starch unexposed or exposed to 2150 lux of light. Each point represents an average of duplicate analyses. □ 4DE exposed, ◊ 4DE unexposed, ⊞ 36.5DE exposed, ◆ 36.5DE unexposed.

tions. Corresponding half-life values were then determined (Table 1). The Arrhenius equation is

$$k = k_o e^{-(Ea/R)/T}$$

where Ea/R is the slope, and ln k_0 is the intercept of the relationship between the natural log of k and 1/T in Kelvin. The Arrhenius equations determined, along with the respective r^2 for each hydrolyzed starch evaluated, are also presented. For a first-order reaction, the half-life is determined at a specific temperature by the equation

$$t_{1/2} = 0.693/k.$$

where k is the rate constant at a specific temperature and $t_{1/2}$ is the half-life. The degradation rate for the control of carrot coagulum spray dried alone was experimentally determined only at 21°C because of the rapid loss of carotenes. Rate constants for the control were 1.34 × 10^{-2} hr^{-1} for α-carotene and 1.22 × 10^{-2} hr^{-1} for β-carotene. The control product degraded to a brown/gray color in only 4 days at 21°C.

All hydrolyzed starches increased half-lives of both α- and β-carotene at 21°C compared to the control. The shelf life of carrot powder was extended by encapsulating with any of the hydrolyzed starches evaluated. Encapsulation of carrot carotenes with hydrolyzed starches increased the half-life 70 to 220 times over the control depending on the hydrolyzed starch used. The beneficial effect of encapsulation as a means to prevent degradation of spray dried carotenes during storage is believed to be due to the physical barrier provided to oxidation.

At all temperatures, hydrolyzed starch of 36.5DE provided the best protection from both α- and β-carotene degradation followed by 15DE, 25DE and 4DE. The less effective encapsulating properties of 4DE hydrolyzed starch were in agreement with results reported by O'Boyle et al. (1992) and Anandaraman and Reineccius (1986). Hydrolyzed starch of 4DE may provide low carotene stability because it contains a large proportion of long chain saccharides that cause the barrier to be inflexible and more permeable to oxygen. A nonlinear relationship between hydrolyzed starch DE and stability characteristics may be ex-
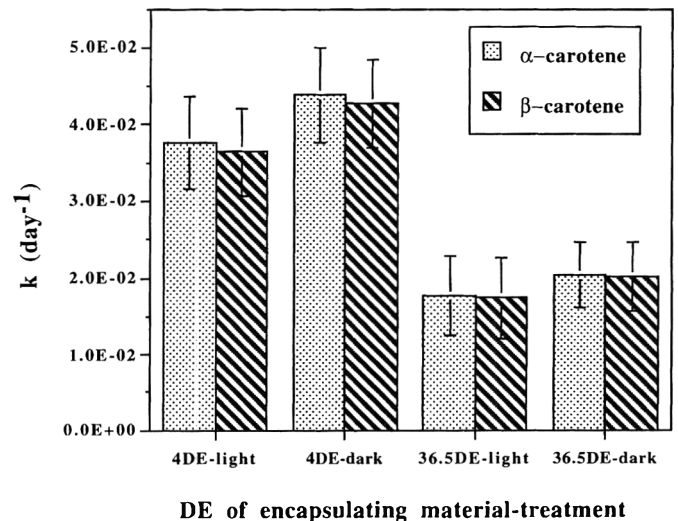

Fig. 5—Comparison of first-order rate constants (k) for 4DE and 36.5DE spray-dried encapsulated carrot carotenes unexposed or exposed to 2150 lux of light at 45°C. Bars indicate 95% confidence intervals.

DE of encapsulating material-treatment

plained by the ways in which starches of varying DE were hydrolyzed. Hydrolyzed starch of 25DE was produced by acid hydrolysis exclusively while the remaining were hydrolyzed by a combination of enzymes and acid. This difference could cause inclusion complexes with aliphatic linear compounds (Wyler and Solms, 1982). During the manufacture of hydrolyzed starch, small quantities cf lipids or fatty acids can become occluded in the starch, and may not be removed by further purification. If occluded lipids have become oxidized, free radicals in the matrix would be generated during storage. Thus differences in degradation rates of carotenes in the encapsulated carrot powders may have been due to differences in the proportion of occluded lipids.

Table 1—Arrhenius equations and half-life values for major carrot carotenes encapsulated with various DEs of hydrolyzed starch[a]

DE	α-carotene degradation (hr^{-1})	β-carotene degradation (hr^{-1})	α-carotene half-life at 21°C (days)	β-carotene half-life at 21°C (days)
4	$k=8.39\times10^9 \cdot e^{-9.23\times10^3/T}$ $r^2=0.992$	$k=5.35\times10^9 \cdot e^{-9.09\times10^3/T}$ $r^2=0.989$	149	145
15	$k=1.01\times10^9 \cdot e^{-8.77\times10^3/T}$ $r^2=0.986$	$k=3.93\times10^8 \cdot e^{-8.47\times10^3/T}$ $r^2=0.963$	258	237
25	$k=4.41\times10^{10} \cdot e^{-9.82\times10^3/T}$ $r^2=0.974$	$k=4.03\times10^{10} \cdot e^{-9.79\times10^3/T}$ $r^2=0.966$	210	209
36.5	$k=2.18\times10^{11} \cdot e^{-1.05\times10^4/T}$ $r^2=0.983$	$k=1.18\times10^{11} \cdot e^{-1.03\times10^4/T}$ $r^2=0.966$	458	431
control	N.D.[b]	N.D.	2.2	2.4

[a] T is in degrees Kelvin
[b] N.D. = Not determined

The spray-dried carrot powder contained some carotene material on the outer surfaces. Surface carotene should be minimized since it is less protected from oxidation than encapsulated carotene. Analysis of surface carotene (percent of total α- or β-carotene) indicated that differences in stability of encapsulated carotenes were not due to differences in carotene retained on the surface. Surface carotenes for 4DE, 15DE, 25DE and 36.5DE encapsulated carrot powders were 11, 23, 29 and 14% α-carotene and 13, 26, 32 and 16% β-carotene, respectively. Although 15DE hydrolyzed starch provided greater stability as indicated by the lower degradation rates of the major carrot carotenes, it had a higher proportion of surface carotene than 4DE. The reason for varied amounts of surface carotene is not known. Moisture content ranged from 2.74 to 3.42% (wb) and water activities ranged from 0.154 to 0.178 in the four carrot powders. These small differences are not enough to explain differences in stability.

Effect of carrier level

Hydrolyzed starch of 15DE was selected for further evaluation of effects of carrier level due to its better protection of carotenes over 4DE and 25DE in preliminary experiments. The powder encapsulated with 15DE hydrolyzed starch was selected over 36.5DE because its lower hygroscopicity would result in higher spray drier yields. The critical moisture point for caking was decreased by using 15DE hydrolyzed starch. This increases the range of relative humidities at which the carrot powder or its product application could be stored without caking (Subramaniam, 1984; Lloyd and Nelson, 1984).

The effects of encapsulating with different levels of 15DE carrier on α- and β-carotene stability were compared (Fig. 6). The degradation rate decreased by ≈75% when the in-feed level of 15DE hydrolyzed starch was increased from 10 to 25%. The recommended total solids level for spray drying is 40% (Miller, 1993). Therefore, with 13.5% solids carrot coagulum 25% hydrolyzed starch was the maximum practical usage level. Research has also shown that the highest possible carrier level should be used to obtain the greatest flavor retention during spray drying (Reineccius and Coulter, 1969; Zilberboim et al., 1986).

The favorable effect of decreased degradation rate with increased proportion of carrier may be due to a corresponding decrease in surface carotene. Evaluation of the amount of surface carotene indicated that as the proportion of 15DE hydrolyzed starch increased, the amount of surface α- and β-carotene decreased (Table 2). Surface carotene differences did not explain differences in stability of powders when encapsulated with various DE. The proportion of surface carotene appears to be a factor in carotene stability when the same DE is used at different levels. Similar findings were reported by Bhandari et al. (1992) when investigating encapsulated citral and linalyl acetate flavorings. They reported that decreased surface volatiles were associated with increasing the solids content of the in-feed emulsion. They also found that at higher in-feed solids, the particle size increased, thereby giving less washable volatile prod-
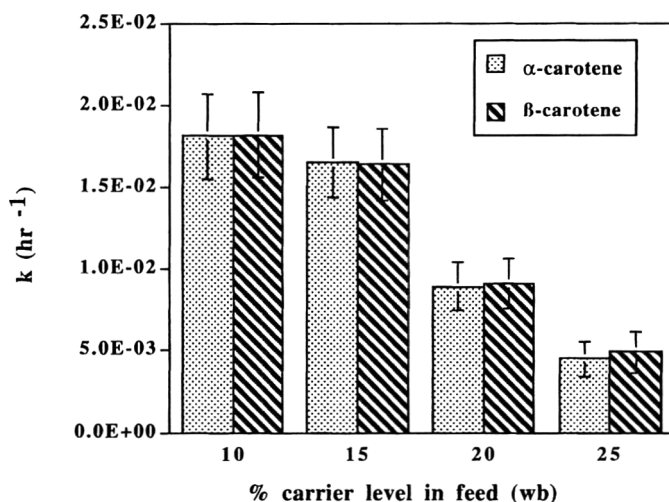

Fig. 6—Comparison of first-order rate constants (k) of α- and β-carotene at 50°C in spray-dried carrot powder when encapsulated with various levels of 15DE hydrolyzed starch. Bars indicate 95% confidence intervals.

ucts. High solids content in the in-feed material additionally would improve drying rate, therefore reducing the production cost.

CONCLUSION

HYDROLYZED STARCH of 36.5DE was superior to 4, 15, and 25DE in improving the retention of α- and β-carotene in spray dried encapsulated carrot powder during storage. All four starches improved the shelf life 70–220 times compared to carrot juice spray-dried alone. Carotene retention could be increased by increasing the carrier level, decreasing storage temperature and packaging in an inert atmosphere. The degradation of α- and β-carotene followed first-order reaction kinetics and an Arrhenius relationship. Light exposure did not accelerate carotene degradation. Autooxidation appeared to be the primary mode of degradation of the carotenes in encapsulated carrot powders. This is the first reported study on the stability of encapsulated α- and β-carotenes from carrots. Encapsulating concentrated carrot solids with hydrolyzed starches provided increased storage stability of α- and β-carotene.

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Table 2—First-order degradation rate constants (k) with corresponding 95% confidence intervals and surface carotene determinations for α- and β-carotene in encapsulated carrot powder encapsulated with various levels of 15DE hydrolyzed starch

% 15DE carrier in feed (wet basis)	α-carotene degradation rate × 10^{-2} (day^{-1})	surface α-carotene (% of total α-carotene)[d]	β-carotene degradation rate × 10^{-2} (day^{-1})	surface β-carotene (% of total β-carotene)[d]
10	1.82 ± 0.258[a]	39	1.82 ± 0.260[a]	37
15	1.66 ± 0.215[a]	34	1.65 ± 0.219[a]	32
20	0.896 ± 0.144[b]	29	0.908 ± 0.153[b]	29
25	0.451 ± 0.107[c]	18	0.493 ± 0.129[c]	19

[a-c] (Different letters within columns indicate a significant difference $p<0.05$)

[d] Values are an average of duplicate analyses

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Texturization of Sweetpotato Puree with Alginate: Effects of Tetrasodium Pyrophosphate and Calcium Sulfate

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————————— ABSTRACT —————————

The effects and optimum levels of tetrasodium pyrophosphate (TSPP), alginate and calcium sulfate (CaSO₄) on physical and sensory characteristics of texturized sweetpotato puree were studied using response surface methodology. Samples were subjected to instrumental texture profile analysis (TPA) and sensory evaluation. Prediction models to describe the effects of ingredients on TPA parameters were used to generate contour plots for optimization using TPA values of baked roots as the limits. The optimum formulations having textural characteristics of the baked roots were within the following identified regions: (a) at 0.12% TSPP, alginate, 0.20–0.25%, and CaSO₄, 0.40–0.70%; (b) at 0.18% TSPP, alginate, 0.20–0.55%, and CaSO₄, 0.22–0.42%. A taste panel scored representative formulations of the optimum regions similar to the baked roots for color, flavor, texture, and overall acceptability.

Key Words: restructured products, texture profile, response surface

INTRODUCTION

MANY REPORTS on various aspects in production of sweetpotato puree have been published (Collins and Walter, 1992). However, there are few accounts of restructured sweetpotato products (Walter and Hoover. 1984; Hoover et al., 1983; Pak, 1982). We have recently published a report on sweetpotato puree restructured with cellulose derivatives (Truong and Walter, 1994). This cellulose gum-texturized product requires minimum home preparation time and has sensory characteristics of traditionally baked sweetpotatoes. However, due to thermally induced gelling properties of cellulose gums, the product retains its texture only at elevated temperature (>50°C). Therefore, it has to be consumed hot, and this may be a limitation of the developed technology.

Alginate is different from cellulose gums in that it forms chemically, rather than thermally, induced gels. The gel network is formed by inter-molecular association of polyvalent cations such as calcium with the polyguluronate sites of alginate molecule (Sime, 1984). Under suitable conditions, alginate gelation can take place at room temperature, resulting in mechanically and thermally stable gels. The alginate/calcium binding technology has proven useful for production of restructured products from fruits and muscle foods (Luh et al., 1976; Hannigan, 1983; Mandigo, 1986; Schmidt and Means, 1986; Pelaez and Karel, 1981). However, there is limited information on its applicability in food systems with high starch content such as sweetpotato puree.

Several factors including pH, temperature, type, and concentration of alginates; calcium salts; and sequestrants used to bind the amount of available calcium affect the gelling reaction (Imeson, 1990). Interactions between sodium alginate, calcium carbonate, and organic acids affecting textural properties of the products have been studied in restructured meat systems (Johnson et al., 1990; Means et al., 1987; Shand et al., 1993; Trout et al., 1990) and fabricated fruits (Kaletunc et al., 1990; Nus-

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sinovitch and Peleg, 1990). Effects of endogenous available calcium on alginate gelation were not studied. Furthermore, investigation on optimization of these parameters using response surface methodology (RSM) has been limited. Using RSM, Mouquet et al. (1992) obtained the optimum ingredient concentrations and processing treatments for mechanical and thermal stability of alginate texturized mango pulp. However, sensory characteristics of the product were not determined.

Our objectives were to (1) explore the feasibility of alginate texturization of sweetpotato puree into restructured products, (2) determine the effects and optimum levels of calcium sequestrant, alginate and calcium salt on textural properties using RSM and (3) evaluate sensory characteristics of combinations which fell in the optimum regions of contour plots, as compared with traditionally baked sweetpotatoes.

MATERIALS & METHODS

Experimental design

Based on preliminary experiments, independent variables affecting texture of restructured sweetpotatoes were tetrasodium pyrophosphate (TSPP) (X1), alginate (X2) and calcium sulfate (X3). A rotatable design with three independent variables (Cochran and Cox, 1957; Mullen and Ennis, 1979) was adopted. Each independent variable had five levels with a central value, and intervals between levels were selected according to preliminary studies: X1 = 0, 0.06, 0.12, 0.18, and 0.24% of the formulation; X2 = 0.20, 0.40, 0.60, 0.80, and 1.00%; and X3 = 0.15, 0.30, 0.45, 0.60, and 0.75%. The experimental design required 15 treatment combinations, with the center point replicated five times (T-15 to T-19). Coded variables and actual percentages of ingredients used in the formulations are shown in Table 1. The experiment was performed with two replications. Dependent variables were the parameters of the instrumental texture profile analysis (TPA) which included fracturability, hardness, cohesiveness, adhesiveness, springiness, and gumminess.

Preparation of puree

Jewel cultivar sweetpotatoes were utilized. The roots were cured and stored at 13–16°C and 80–90% relative humidity (RH) for 5 mo prior

Table 1—Coded and uncoded variables in the treatment formulations of sweetpotato puree texturized with alginate/calcium

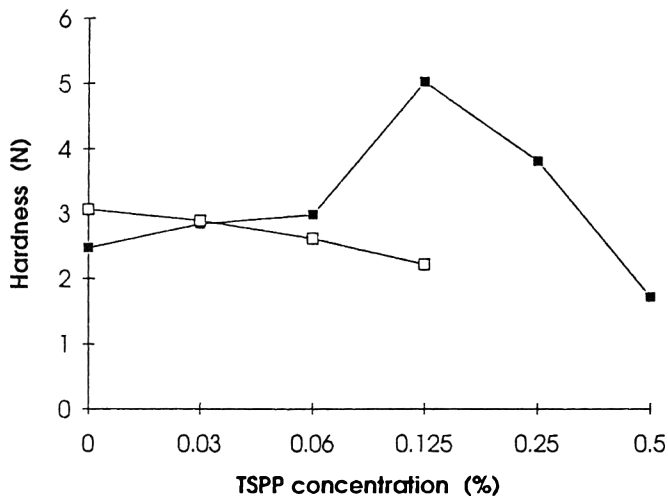| Treatment combination	Code			%		
	X1	X2	X3	TSSP	ALG	CaSO₄
1	−1	−1	−1	0.06	0.4	0.3
2	−1	−1	1	0.06	0.4	0.6
3	−1	1	−1	0.06	0.8	0.3
4	−1	1	1	0.06	0.8	0.6
5	1	−1	−1	0.18	0.4	0.3
6	1	−1	1	0.18	0.4	0.6
7	1	1	−1	0.18	0.8	0.3
8	1	1	1	0.18	0.8	0.6
9	1.682	0	0	0.24	0.6	0.45
10	−1.682	0	0	0	0.6	0.45
11	0	1.682	0	0.12	1	0.45
12	0	−1.682	0	0.12	0.2	0.45
13	0	0	1.682	0.12	0.6	0.75
14	0	0	−1.682	0.12	0.6	0.15
15	0	0	0	0.12	0.6	0.45
16	0	0	0	0.12	0.6	0.45
17	0	0	0	0.12	0.6	0.45
18	0	0	0	0.12	0.6	0.45
19	0	0	0	0.12	0.6	0.45

Fig. 1—Effect of TSPP and CaSO₄ concentration on gel hardness of restructured sweetpotato texturized with 0.5% alginate. (□) 0.15% CaSO₄, (■) 0.45% CaSO₄.

Table 2—Mean values of instrumental texture profile parameters of treatment formulations and baked sweetpotato roots (control) measured at 25°C

Treatment combination	Fracturability (N)	Hardness (N)	Cohesiveness (%)	Adhesiveness (mJ)	Springiness (%)	Gumminess (N)
1	1.13	3.00	49.43	10.91	96.39	1.49
2	1.53	3.85	32.53	6.55	44.31	1.23
3	2.19	4.62	32.23	9.59	53.20	1.49
4	2.60	5.53	24.09	7.16	32.49	1.32
5	4.27	6.47	14.52	4.10	27.42	0.94
6	9.26	10.80	10.20	1.27	26.93	1.10
7	8.44	9.71	15.97	5.02	40.98	1.55
8	19.73	19.86	11.31	0.23	30.94	2.25
9	10.58	10.75	11.01	1.51	30.82	1.17
10	0.37	3.04	67.99	12.82	117.76	2.06
11	21.18	19.72	9.16	0.18	31.12	1.79
12	3.86	6.41	13.21	2.85	23.68	0.82
13	9.2′	12.51	11.24	0.84	29.49	1.35
14	2.09	3.92	44.86	15.77	119.67	1.76
15	13.54	13.64	10.51	0.93	26.61	1.43
16	8.34	12.49	11.82	1.14	28.45	1.45
17	11.47	13.76	11.55	0.92	29.45	1.54
18	13.93	13.16	10.19	0.77	31.50	1.34
19	10.90	14.27	10.82	0.99	29.32	1.53
Baked roots	4.10	8.70	7.80	1.23	13.52	0.68

to use. The puree was prepared as previously described (Truong and Walter, 1994) and had the following composition (fwb): dry matter, 21.92%; alcohol-insoluble solids, 9.40%; starch, 4.14%; fructose, 1.56%; glucose, 1.62%; sucrose, 7.17%; and maltose, 6.45%.

Preparation of restructured sweetpotatoes

Texturization of sweetpotato puree was carried out by mixing the sweetpotato puree with other ingredients in an electronic chopper (Model UMC5, Stephan Co., West Germany) at 1800 rpm for 3 min with interval interruptions. Each formulation (1 kg) contained, in addition to sweetpotato puree, 60g sucrose and amounts of TSPP (Rhone-Poulenc, Shelton, CT), alginate (type Manugel-DMB, Kelco, Rahway, NJ) and calcium sulphate dihydrate (Merck and Co., Inc., Rahway, NJ) equivalent to levels indicated (Table 1). The 6% sucrose added to the formulation was intended to raise the sugar level of puree to attain sweetness usually preferred for baked sweetpotato (Truong and Walter, 1994). Ingredients were mixed with the puree according to the following sequence: first, TSPP pre-mixed in 20g sucrose, followed by alginate-sucrose mix, and calcium sulfate suspended in 20 mL of water. Mixing time was 60 sec after adding each ingredient mix. The blended mixture was immediately extruded into 5.5 cm diameter sausage casings and clipped to form rolls of about 10 cm in length, and the ends fastened. The rolls were aged for 24 hr at 4°C, frozen, and stored at −20°C.

Sensory evaluation

Before baking, sausage casings of frozen restructured sweetpotatoes were removed. Unthawed samples were baked in a conventional oven at 204°C for 15 min, immediately cut into 2.5 cm thick slices perpendicular to the long axis, each slice placed in ≈120 mL glass jars and kept at 70°C until served (Truong and Walter, 1994). The elapsed time between sample preparation and evaluation was about 1–2 hr. The product texture was stable under these conditions. The temperature of samples was 55–65°C when evaluated by panelists. For baked sweetpotato roots, stored roots of fairly uniform shape and size (ca. 6.5–7.5 cm in diameter) were selected, carefully washed, air dried, wrapped in aluminum foil, and baked at 204°C for 90 min. Middle portions of baked roots were cut into slices similar to those of restructured sweetpotatoes.

Samples of selected formulations and baked roots were subjected to an acceptability test by a 30-member, untrained panel consisting of faculty, staff, and graduate students from the Department of Food Science at North Carolina State Univ. At each testing, panelists were asked to evaluate four samples in a random order for color, flavor, texture and overall acceptability on a 9-point hedonic scale (9 = like extremely, 1 = dislike extremely). All panel sessions were conducted in sensory panel booths under fluorescent light.

Physical measurements

Instrumental texture profile parameters were determined at 25°C following the TPA procedure. Fracturability, hardness, adhesiveness, co-

Table 3—Analysis of variance of overall main effects of ingredient concentration, coefficients of determination (R²) and significance of the full regression models for TPA values

Independent variable	F-ratio					
	Fracturability	Hardness	Cohesiveness	Adhesiveness	Springiness	Gumminess
TSPP	18.97**	22.10**	149.22**	50.86**	27.27**	2.80
ALG	16.76**	19.24**	3.61	0.90	0.18	18.13**
CaSO₄	6.23*	12.05**	38.15**	46.68**	25.25**	0.19
R²	0.87	0.90	0.97	0.94	0.91	0.80
F-ratio for total regression	6.71**	8.73**	32.29**	16.37**	9.95**	3.96*

* Significant at P < = 0.05; ** Significant at P < = 0.01.

hesiveness, and gumminess were determined as described by Bourne (1978). Springiness was calculated as a proportion of the compression distance recovered between the first and second compression (Montejano et al., 1985). Cohesiveness and springiness were expressed in percentage. Baked samples were cooled at room temperature for several hours and cut into 2 cm cubes (about 8–9g). Cubes of restructured and baked root samples were weighed and then subjected to 75% double compression and relaxation using an Instron Universal Testing Machine (Model 1122, Instron Inc., Canton, MA) fitted with a 50 kg-load cell which was attached with a plunger having a 5.7 cm diameter compression anvil. The measurements were performed with a crosshead speed of 10 cm/min and a chart speed of 20 cm/min. At least four measurements were taken for each sample of the formulated sweetpotatoes. Baked roots of similar size and shape were used, and samples were taken at the middle part of each root. Data were expressed in unit of texture profile parameters per 10-g sample.

Hunter color values (L, a, b) were determined from reflectance measurements using a Spectrogard color system (Pacific Scientific, Silver Spring, MD) with daylight illuminant.

Calcium determination

Calcium content was determined following the spectrophotometric procedure (Gindler and King, 1972).

Statistical analysis

All analyses were performed using the Statistical Analysis System (SAS Institute, Inc. 1989). A second order response surface model which had linear, quadratic, and all interaction terms for the three independent variables was fitted to the TPA data of the 19 treatment combinations. Dependent variables were the TPA parameters including fracturability, hardness, cohesiveness, adhesiveness, springiness, gumminess and chewiness. Independent variables which were found significant at P < 0.05 in the full model were retained in the reduced models. The best final model was obtained following the step-wise regression procedure described by Draper and Smith (1981). Models that were significant (P < 0.05) with R² > 0.70 were used to generate contour plots for each TPA

Table 4—Best selected prediction models for instrumental texture profile parameters[a]

Dependent variable	Prediction equation	R^2
Fracturability	$y = -33.75 + 174.17X_1 + 16.06X_2 + 81.64X_3 - 488.40X_1^2 - 76.20X_3^2$	0.79
Hardness	$y = -27.22 + 164.62X_1 + 13.19X_2 - 71.21X_3 - 489.47X_1^2 - 63.65X_3^2$	0.82
Cohesiveness	$y = 150.31 - 864.80X_1 - 44.99X_2 - 215.61X_3 + 293.75X_1X_2 + 1999.78X_1^2 + 192.69X_3^2$	0.96
Adhesiveness	$y = 38.73 - 153.76X_1 - 93.22X_3 + 440.08X_1^2 + 83.08X_3^2$	0.93
Springiness	$y = 253.29 - 1017.37X_1 - 551.94X_3 + 3049.82X_1^2 + 491.19X_3^2$	0.83
Gumminess	$y = 3.22 - 20.05X_1 - 0.90X_2 - 2.31X_3 + 17.39X_1X_2 + 17.92X_1X_3$	0.72

[a] X_1 = TSPP; X_2 = alginate; X_3 = $CaSO_4$.
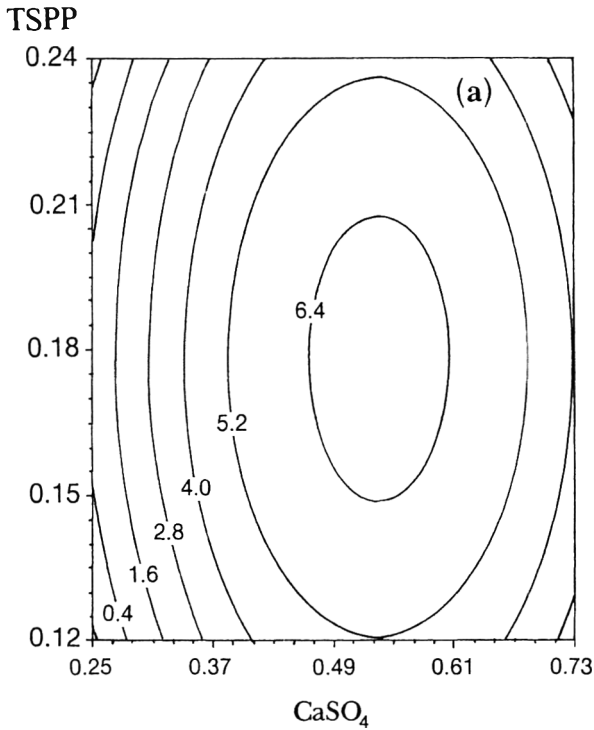
Fig. 2—Contour plots of (a) fracturability, (b) cohesiveness, and (c) gumminess of restructured sweetpotato as a function of TSPP and $CaSO_4$ concentration at 0.2% alginate.
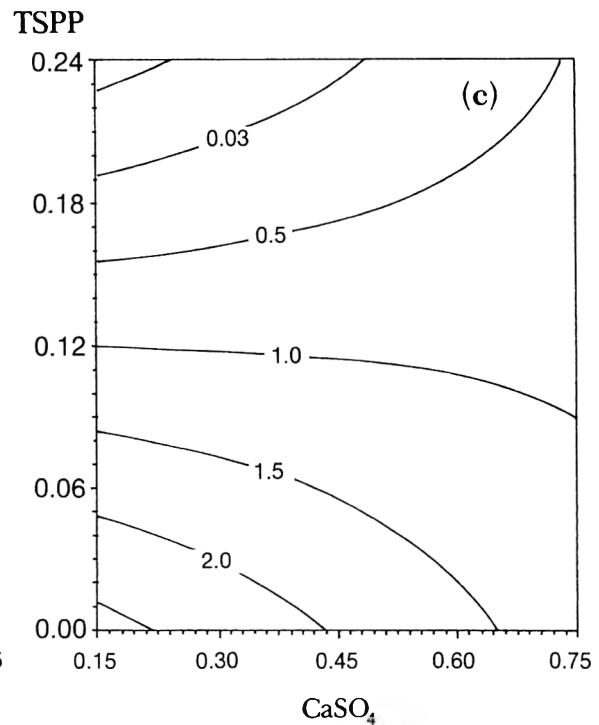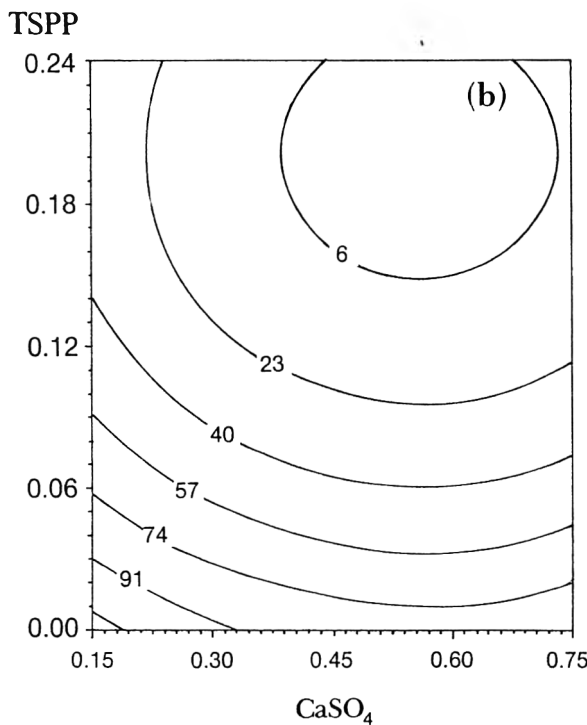
parameter as a function of two variables, while the other variable was held constant.

The baked roots served as the control, and ranges of their TPA values were used to set limits in each contour plot for identification of an ac-ceptable region. Contour plots for TPA parameters were superimposed, and regions of overlap were shaded. The overlapping area indicates the treatment combinations which are expected to have textural character-istics similar to baked roots.
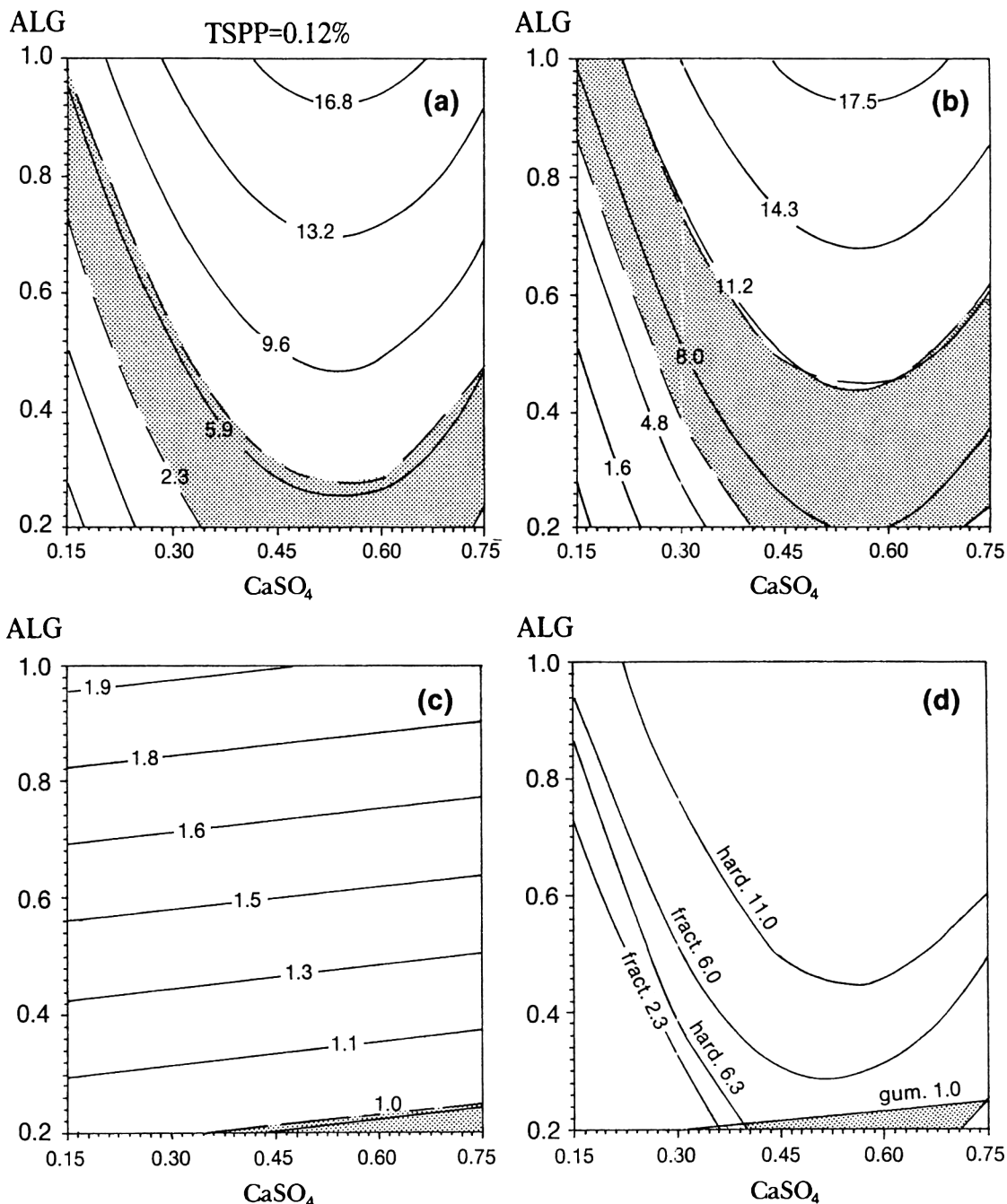
Fig. 3—Contour plots of (a) fracturability, (b) hardness, (c) gumminess of restructured sweetpotato as a function of alginate and CaSO₄ concentration at 0.12% TSPP with shaded regions covering the limits of the control, and (d) optimum (shaded) region obtained by superimposing (a), (b) and (c).

RESULTS & DISCUSSION

Texturizing conditions

The calcium content of sweetpotato puree was 33.71 mg/100g which was in the range of 17–45 mg reported for sweetpotato roots (Kays, 1992). Picha (1985) also obtained a similar result of 30 mg calcium/100g in 'Jewel' sweetpotato roots. Available calcium can readily interact with added alginate, resulting in premature gel formation and, consequently, gel softness (Imeson, 1990). Therefore, TSPP was used as a calcium sequestrant and was dispersed in puree prior to addition of alginate and calcium sulfate. However, at a given alginate concentration (e.g. 0.50%, w/w) increasing TSPP concentration in the formulation could result in an increase or decrease in gel hardness (Fig. 1), depending on the amount of calcium sulfate available to interact with alginate. Optimal concentrations of these ingredients

should be determined to obtain gel characteristics suitable for a given texturized product.

In preparation of the designed formulations (Table 1), we observed that the setting time before gelation varied from <10 min to 1–2 hr. In addition, the source of calcium sulfate had an effect on gelation time. The gel hardness reached maximum after aging at 4°C for 24 hr. According to Nussinovitch and Peleg (1990) the physical and chemical equilibrium of alginate gels can take from 6 to >48 hr. However, measurements of mechanical properties of the gels can be performed after 24 hr since changes after that are relatively small.

Freezing and thawing of the sweetpotato puree-alginate gel resulted in lowering of its hardness and fracturability values by 30% and 50%, respectively. However, cohesiveness, adhesiveness, and springiness were unchanged. The alginate gel is thermally stable (Imeson, 1990). Baking the sweetpotato-
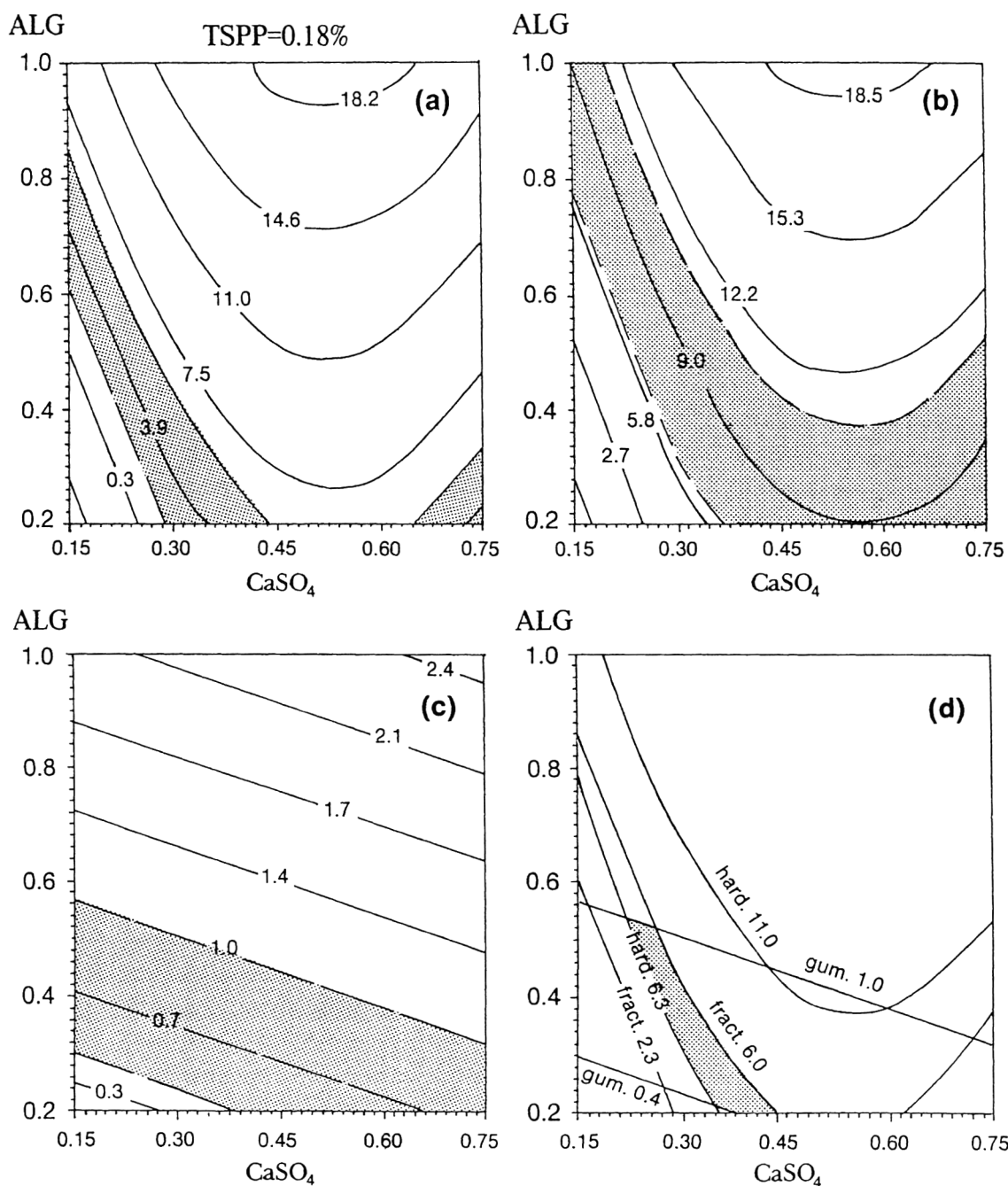
Fig. 4—Contour plots of (a) fracturability, (b) hardness, (c) gumminess of restructured sweetpotato as a function of alginate and CaSO₄ concentration at 0.18% TSPP with shaded regions covering the limits of the control, and (d) optimum (shaded) region obtained by super-imposing (a), (b) and (c).

alginate gel at 204°C for 15 min had no effect on any TPA parameters.

Model fitting and mapping of contour plots

The mean values of TPA parameters for the 19 treatment combinations and baked sweetpotato roots are summarized (Table 2). Multiple regression analysis resulted in significant full models ($P < 0.05$) for all dependent variables. The TSPP and $CaSO_4$ concentrations had significant main effect ($P < 0.05$) on all TPA parameters except gumminess (Table 3). On the other hand, the main effect of alginate levels was significant only for fracturability, hardness, and gumminess. Most interaction terms of the independent variables in the full model showed no significant effect on TPA parameters (data not shown).

Stepwise regression analysis resulted in reduced models (Table 4). These were not significantly different ($P < 0.05$) from the full model, based on the F-test, and were used to generate contour plots for TPA parameters. A contour plot of fracturability of restructured sweetpotatoes containing 0.20% alginate as a function of TSPP and $CaSO_4$ concentrations is shown (Fig. 2a). A similar contour plot pattern was obtained for hardness (graph not shown). Increasing TSPP and $CaSO_4$ concentrations resulted in an increase in these firmness parameters with maximal values at 0.18% TSPP and 0.56% $CaSO_4$.

A contour plot for cohesiveness as a function of TSPP and $CaSO_4$ at 0.20% alginate was compared (Fig. 2b). Unlike fracturability and hardness, cohesiveness decreased as concentration of TSPP and $CaSO_4$ increased. Contour plots for adhesiveness and springiness exhibited the same pattern (graphs not shown). Optimal TSPP and $CaSO_4$ levels for these TPA parameters were

also attained at about 0.14–0.17% and 0.56%, respectively. A formulation containing these optimal levels (0.16% TSPP, 0.56% CaSO₄) and 0.20% alginate which was F-0.2Alg was prepared for including in acceptability tests for comparison.

Gumminess, the product of hardness and cohesiveness (Bourne, 1978) was linearly related to TSPP, alginate and CaSO₄ concentrations (Table 4). Contour plots of gumminess at 0.20% alginate (Fig. 2c) exhibited different patterns from other TPA parameters. Increasing both TSPP and CaSO₄ concentrations resulted in a corresponding decrease in gumminess of the formulations having less than 0.12% TSPP. However, with TSPP levels >0.12%, reduction in gumminess could be obtained by lowering the amount of CaSO₄.

In order to observe the effects of alginate and CaSO₄ on product texture, contour maps of TPA parameters were plotted at constant TSPP levels. At TSPP concentrations of 0.12% (Fig. 3a and 3b) and 0.18% (Fig. 4a and 4b), fracturability and hardness increased with increasing levels of both alginate and CaSO₄. Gumminess values decreased with reduced alginate levels (Fig. 3c and 4c). Note that changes in gumminess as affected by CaSO₄ concentration were dependent on TSPP level. At 0.12% TSPP, lowering CaSO₄ concentration resulted in increased gumminess (Fig. 3c). A reversed trend was exhibited at 0.18% TSPP (Fig. 4c), at which low gumminess would result from reducing CaSO₄ concentration.

Attaining the optimum treatment combinations

We previously reported that the instrumental fracturability, hardness and gumminess correlated (R = 0.65–0.74) with sensory scores of texture notes on simulated baked sweetpotatoes (Truong and Walter, 1994). Though correlations were about marginal to be used as predictors of sensory scores as stated by Bourne (1982), we used those parameters to develop optimum formulations with textural characteristics of a control. The following TPA values were within two standard deviations of means for the control, the 'Jewel' baked roots (n = 30): fracturability, 2.3–6.0N, hardness, 6.3–11.0N and gumminess, 0.4–1.0N. These values were used to set constraints for TPA parameters of the formulated sweetpotato. The shaded areas (Fig. 3a, 3b, 3c, 4a, 4b, 4c) represent values for respective TPA parameters corresponding to limits specified. Figure 3d was obtained by super-imposing the 0.12% TSPP contour maps of fracturability (Fig. 3a), hardness (Fig. 3b) and gumminess (Fig. 3c). In the same manner, Figure 4d was generated by overlaying the 0.18% contour maps (Fig. 4a, 4b, 4c). The shaded regions (Fig. 3d, 4d) represent all treatment combinations that would result in products with textural characteristics within the limits of baked sweetpotato roots. At 0.12% TSPP, the optimum formulations should contain: alginate, 0.20–0.25% and CaSO₄, 0.40–0.70%. At 0.18% TSPP, the alginate and CaSO₄ levels should be shifted to 0.20–0.55% and 0.22–0.42%, respectively.

Note that T-12 and T-5 (Table 1) fall within the overlapping regions identified in Fig. 3d and 4d, respectively.

Product acceptability

Samples of T-5, T-12, and F-0.2Alg, together with the baked roots, were subjected to acceptability sensory tests. Color, flavor, texture, and overall acceptability of the baked roots were scored at 7.1, 6.2, 6.9, and 6.4, respectively. The T-5 and T-12 formulations scored the same (P < 0.05) for color (7.4–7.5), flavor (6.0–6.7), texture (6.6–6.9), and overall acceptability (6.2–6.8). However the F-0.2Alg samples had the lowest scores for flavor (5.2), texture (6.2), and overall acceptability (5.5), which were different from the control. Several panelists reported a slightly bitter after-taste in the F-0.2Alg formulation. This was probably attributable to its high level (0.56%) of calcium sulfate.

Product color

The baked roots had L, a, and b values of 59.7, 21.7, and 45.3, respectively, similar to values previously reported (Truong and Walter, 1994). The beta-carotene content of baked 'Jewel' roots has been reported as high as 6.26–7.99 mg/100g sample (Wu et al., 1991). The T-5, T-12, and F-0.2Alg formulations had the same values for L (58.7–59.1), a (21.2–21.6), and b (38.8–39.3). The difference in degree of lightness (L value) and redness (a values) between formulated samples and baked roots was not significant (P < 0.05). However, the b values for the formulated were significantly lower than those for roots. Thus, restructured products had a lower intensity of yellowness than baked roots.

CONCLUSIONS

THE ALGINATE/CALCIUM SYSTEM is excellent for texturizing sweetpotato puree. The restructured system required minimal preparation and yet had the textural and flavor acceptability equal to traditionally baked sweetpotatoes. Moreover, such texturization could be applied to produce other types of processed products from sweetpotatoes. This research demonstrates the application of response surface statistical design together with instrumental texture profile parameters to attain optimum formulations for alginate-texturized products. Using this method, the number of samples subjected to sensory evaluation can be substantially reduced.

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Screening Potato Starch for Novel Properties Using Differential Scanning Calorimetry

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──────────── ABSTRACT ────────────

THERMAL and other physicochemical properties of starch from 42 potato genotypes were studied to find those with unique properties for food use, and to analyze relationships between thermal and other physicochemical properties. Onset and peak transition temperatures and gelatinization enthalpy intercorrelated. Transition temperatures intercorrelated with pasting temperature using a Brabender Visco-amylograph. Gelatinization enthalpy correlated with Brabender pasting temperature and peak paste viscosity, and onset temperature correlated with phosphorus content. Genotype E55-35 with highest onset and peak transition temperatures also had highest phosphorus and peak Brabender viscosity. DSC might be useful for rapidly screening samples of <1g starch for such. Potato starch DSC characteristics did not correlate with amylose, intrinsic viscosity, or water-binding. For 10 genotypes from successive years, correlations were observed for pasting temperature ($r = 0.83$), phosphorus content ($r = 0.80$), and stability ratio ($r = 0.66$). Direct comparison between samples from consecutive years showed good reproducibility for amylose, but not for phosphorus or pasting.

Key Words: potato, starch, genotypes, transition temperatures, gelatinization enthalpy

INTRODUCTION

FOOD PROCESSORS AND GROWERS are interested in identification or development of crop germplasm which yields native starches with unique physico-chemical characteristics. Such starches may supplant some chemically-modified starches in formulated foods or open markets for new applications of starch. The grower in a highly competitive market which has depressed crop prices seeks germplasm which may create higher-value niche markets. This has resulted in the genetic manipulation of crops such as potatoes to alter starch characteristics (Shewmaker et al., 1994).

Potato starch has several inherent, desirable characteristics. These include swelling power that results in high viscosity pastes. Phosphate groups esterified to the amylopectin fraction of potato starch contribute to the high viscosity and also to high transparency, water-binding capacity and freeze-thaw stability (Craig et al., 1989; Swinkels, 1985). The hydration behavior of potato starch differs from cereal starches, and suggests its application in formulated foods requiring high stability and long shelf life (Yakubu et al., 1990).

Many methods for characterizing starch have been developed, which could be used for screening large numbers of genotypes for unique properties. Brabender Viscoamylography data for starch from Red Pontiac tubers suggested the use of unmodified starch as a replacement for chemically crosslinked starch (McComber et al., 1988). Chemical composition of potato starch, especially phosphorus and amylose content, correlated with certain Visco-amylograph results such as peak viscosity and ratio of minimum viscosity during paste breakdown to peak viscosity (Wiesenborn et al., 1994).

Differential scanning calorimetry (DSC) has been applied in many studies of thermal properties of starch since its first use by Stevens and Elton (1971). The method requires <1 g of sample; thus, it could be useful for screening starch samples from large numbers of genotypes. Starch transition temperatures and gelatinization enthalpies by DSC may be related to characteristics of the starch granule, such as degree of crystallinity (Krueger et al., 1987). This is influenced by chemical composition and in turn helps determine thermal and other physical characteristics. Starch from genetically modified potato genotypes which contained less amylose and phosphorus and demonstrated low paste viscosity compared to controls, also exhibited lower transition temperatures and gelatinization enthalpy by DSC (Shewmaker et al., 1994).

Our objective was to examine the suitability of DSC for screening starch from potato genotypes for unique characteristics. The relationships of transition temperatures and gelatinization enthalpy to selected physico-chemical characteristics was examined. We also expected this study might help identify genotypes containing unique starch, and aid in understanding relationships of composition, structure and function.

MATERIALS & METHODS

Source of potato starch tubers

Genotypes (42) of potato tubers which were grown in the 1991 season were obtained from the USDA Potato Research Laboratory in East Grand Forks, MN. The potato tubers were stored for 2 mos at $\approx 7°C$ before starch extraction.

Starch isolation and purification

Starch was isolated and purified using a modification of the procedure of Wiesenborn et al. (1994). Tubers (2.5 kg total wt.) were rinsed in tap water to remove all dirt, peeled and cut into 2–3 cm cubes. Distilled water was used in all subsequent operations. Tubers were then macerated at low speed (Blendor 7010 Model 31BL91, Waring, New Hartford, CT) with water (400g tuber: 400g water) for 45 sec. The mixture was allowed to stand 20 min, then the liquid and remaining suspended solids were decanted away from the sediment and macerated for an additional 45 sec. The second mixture was added back to the sediment, and allowed to stand at room temperature for 40 min. The liquid was decanted away from the sediment and discarded.

The sediment contained some non-starch material which was removed as follows: A suspension of sediment in 0.5 L water was passed through a U.S. No. 20 sieve. The material retained by the sieve was washed on the sieve with about 0.2 L water, resuspended in 0.4 L water, macerated for 45 sec, then again passed through a U.S. No. 20 sieve. Solids retained on the sieve were rinsed on the sieve with 1–2 L water then discarded. Starch in the filtrate and rinse water was allowed to settle 30–45 min, and the liquid decanted off and discarded. The starch was then resuspended in 1 L water and passed through a U.S. No. 60 sieve. The retained solids were again rinsed on the sieve with 1–2 L of water and discarded. Starch in the filtrate and wash water was allowed to settle 30–45 min and the liquid decanted. The starch was resuspended in 0.5 L of water and the settling and decanting steps repeated once without sieving.

After drying in a convection oven at <35°C for 1–3 days, the starch was ground with a mortar and pestle to pass a U.S. No. 60 sieve. The dried starch was spread 1 cm deep on a tray then stored at ambient temperature ($\approx 23°C$) and humidity for 2–3 days prior to transfer to airtight storage containers.

Moisture content

Triplicate samples of 2g starch were weighed in aluminum pans, and dried at 95°C and 710–740 mmHg vacuum for 12 hr. Samples then were cooled in a desiccator for 30 min and weighed to ±0.1 mg.

Protein, amylose, and phosphorus

Protein content of purified potato starch was determined in duplicate by the Micro-Kjeldahl method as described in AACC Approved Method 46-13. Amylose content was determined in triplicate using the rapid

Table 1—Thermal properties of starch from 42 potato genotypes as characterized by DSC[a]

	To[b] (°C)	Tp[c] (°C)	Tc[d] (°C)	ΔH[e] (J/g)
Agassiz	63.5 ± 0.15	67.2 ± 0.14	73.2 ± 0.26	17.7 ± 0.13
Atlantic	63.6 ± 0.18	66.2 ± 0.19	70.2 ± 0.24	17.7 ± 0.06
Bintji	62.3 ± 0.51	65.2 ± 0.48	69.7 ± 0.26	17.2 ± 0.00
C.M.C.	63.3 ± 0.28	66.2 ± 0.32	71.3 ± 0.47	17.3 ± 0.07
Eide	63.6 ± 0.34	67.2 ± 0.45	74.0 ± 0.62	17.9 ± 0.08
Erik	65.7 ± 0.25	68.3 ± 0.19	72.2 ± 0.19	17.3 ± 0.23
Gemchip	64.2 ± 0.24	66.9 ± 0.24	71.5 ± 0.42	18.1 ± 0.12
Irish Cobbler	63.2 ± 0.17	66.0 ± 0.25	70.3 ± 0.45	17.3 ± 0.02
Kennebec	62.8 ± 0.26	65.5 ± 0.37	70.1 ± 0.64	17.5 ± 0.03
Krantz	62.1 ± 0.29	65.5 ± 0.38	71.7 ± 0.37	17.3 ± 0.03
Mainechip	63.2 ± 0.20	66.3 ± 0.29	71.4 ± 0.47	17.0 ± 0.02
Monona	65.4 ± 0.26	68.4 ± 0.29	73.8 ± 0.24	17.4 ± 0.07
Norchip	59.9 ± 0.29	62.9 ± 0.35	67.8 ± 0.65	16.7 ± 0.08
Norking Russ.	63.4 ± 0.53	66.7 ± 0.65	71.8 ± 0.75	17.4 ± 0.03
Red Lasoda	63.5 ± 0.26	66.4 ± 0.27	72.2 ± 0.44	17.8 ± 0.09
Red Norland	63.9 ± 0.12	66.7 ± 0.13	71.4 ± 0.21	17.1 ± 0.10
Red Pontiac	64.7 ± 0.21	67.6 ± 0.31	72.5 ± 0.43	17.1 ± 0.05
Reddale	62.9 ± 0.08	66.0 ± 0.08	72.5 ± 0.22	17.4 ± 0.10
Redsen	64.5 ± 0.06	67.6 ± 0.12	73.0 ± 0.17	17.6 ± 0.05
Russet Burbank	62.6 ± 0.18	65.3 ± 0.21	69.5 ± 0.50	17.0 ± 0.02
Russet Norkota	63.4 ± 0.17	66.3 ± 0.14	71.3 ± 0.19	17.8 ± 0.18
Sebago	65.2 ± 0.06	68.1 ± 0.08	73.3 ± 0.19	17.6 ± 0.09
Shasta	63.8 ± 0.08	67.0 ± 0.14	72.1 ± 0.32	17.1 ± 0.02
Shepody	63.0 ± 0.33	66.2 ± 0.34	71.5 ± 0.44	17.1 ± 0.05
Snowden	61.2 ± 0.36	64.3 ± 0.41	69.8 ± 0.26	16.8 ± 0.13
Superior	65.4 ± 0.26	68.2 ± 0.36	73.2 ± 0.54	17.8 ± 0.10
Tolaas	62.5 ± 0.31	65.2 ± 0.38	69.5 ± 0.65	17.2 ± 0.01
Triumph	63.8 ± 0.12	66.6 ± 0.16	71.4 ± 0.19	17.3 ± 0.05
Waseca	64.5 ± 0.17	67.4 ± 0.22	72.4 ± 0.50	16.8 ± 0.09
A80559-2	63.8 ± 0.05	66.7 ± 0.17	71.7 ± 0.27	17.5 ± 0.10
C69-1A	62.6 ± 0.07	65.7 ± 0.17	71.0 ± 0.37	16.9 ± 0.04
C71-18	63.3 ± 0.15	66.1 ± 0.16	71.2 ± 0.24	17.2 ± 0.04
C71-41	63.1 ± 0.27	66.2 ± 0.30	71.8 ± 0.43	17.1 ± 0.03
C72-93	62.7 ± 0.11	65.6 ± 0.15	70.2 ± 0.27	16.7 ± 0.06
E55-35	66.2 ± 0.36	69.6 ± 0.47	75.4 ± 0.42	17.5 ± 0.03
E55-44	64.8 ± 0.39	67.8 ± 0.46	72.9 ± 0.41	17.9 ± 0.26
GS7232-4	64.6 ± 0.18	67.6 ± 0.27	72.4 ± 0.43	18.1 ± 0.09
ND651-9	63.3 ± 0.05	67.1 ± 0.09	72.5 ± 0.34	16.9 ± 0.15
ND860-2	63.4 ± 0.09	66.4 ± 0.16	71.8 ± 0.35	17.5 ± 0.01
ND1538-1 Russ	61.4 ± 0.40	64.9 ± 0.47	73.1 ± 0.58	16.7 ± 0.02
W870	63.9 ± 0.08	66.5 ± 0.11	70.9 ± 0.30	17.5 ± 0.08
W877	63.3 ± 0.04	67.1 ± 0.08	72.5 ± 0.13	17.6 ± 0.14
Mean ± S.D.	63.5 ± 1.22	66.5 ± 1.21	71.7 ± 1.43	17.3 ± 0.37

[a] Values are average ± standard deviation. Starch-water 30 : 70 (w/w, dry basis, v=0.78). Heating rate of DSC: 10° C/min.
[b] Onset temperature.
[c] Peak temperature.
[d] Conclusion temperature.
[e] Gelatinization enthalpy.

calorimetric method and empirical formula described by Williams et al. (1970). Phosphorus was determined in duplicate using Method B-47 (Corn Industries Foundation, 1985), with samples of ≤0.50 g starch each.

Thermal analysis

Thermal characteristics of potato starch were studied using a Perkin-Elmer Differential Scanning Calorimeter-7 with an attached UNIX Operating System. Starch samples (3–3.5 mg, dry weight) were loaded into aluminum sample pans, and deionized water was added to achieve a starch-water suspension containing 70% water. Sample pans were hermetically sealed and allowed to stand ≥1 hr at room temperature before heating. Samples were then heated at 10°C/min from 40 to 100°C. An empty pan was used as reference. From the curve obtained, the onset temperature (T_o), peak temperature (T_p), conclusion temperature (T_c), and heat of transition (ΔH) were calculated automatically and averaged over three to four trials. The DSC was calibrated with deionized water and indium standards.

Intrinsic viscosity

Intrinsic viscosity was determined using the procedure described by Leach (1963) with an Ubbelohde (Cannon Fenske) viscometer, capillary number 75 at 25°C. A suspension of 0.5 g starch (dry basis) in 50 mL water was cooked in boiling water for 30 min while covering the beaker with a watch glass to prevent evaporation, then cooled to room temperature. A solution containing 0.5% starch was prepared by adding 20 mL 5N NaOH to the cooked suspension, diluted to 100 mL with distilled

water, then shaken vigorously to provide a uniform and lump-free starch dispersion, and centrifuged at 900 × g for 5 min to remove insolubles. Measurement of flow time (T) for samples containing 0.50, 0.33, 0.25, 0.20 and 0.00% starch was repeated until three consecutive measurements were obtained which were reproducible ± 0.2 sec. Flow time of the starch solution was measured with a Wescan automatic viscosity timer (Wescan Instruments Inc., Santa Clara, CA). Intrinsic viscosity was determined by a plot of reduced viscosity vs concentration extrapolated to zero concentration. Reduced viscosity was defined as the ratio of specific viscosity ($T/T_o - 1$) to starch concentration.

Water-binding capacity

Water binding capacity of starch was determined in duplicate according to the procedure of Yamazaki (1953) as modified by Medcalf and Gilles (1965). A suspension of 5 g starch (dry wt. basis) in 75 mL distilled water was agitated for 1 hr on a wrist-action shaker (Burrell, Burrell Corp., Pittsburgh, PA.). The suspension was then centrifuged, the free water decanted from the wet starch, drained for 10 min, and the wet starch weighed.

Pasting characteristics

The starch pasting characteristics were determined with a Brabender Visco-Amylograph Type VA-1B and a 700 cmg cartridge. A suspension of 3.25% (w/w) starch in 450 mL deionized water was heated from 30 to 95°C at 1.5°C/min, held 15 min at 95°C, then cooled to 50°C at 1.5°C/min (McComber et al., 1988). Paste stability ratio was defined as the ratio of viscosity at onset of cooling to peak viscosity prior to cooling. Paste setback ratio was defined as ratio of viscosity at completion of cooling to viscosity at onset of cooling (Wiesenborn et al., 1994).

Statistical analysis

The Statistical Analysis Program System (SAS Institute, Inc., 1990) was used to analyze all data. Least significant differences were computed at P < 0.05.

RESULTS & DISCUSSION

Thermal properties

Results of the DSC characterization of starch from 42 potato genotypes were compared (Table 1). Onset temperatures of gelatinization ranged from 59.9 to 66.2°C, and peak temperatures from 62.9 to 69.6°C. Genotypes Norchip and E55-35 had the lowest and highest transition temperatures, respectively. High transition temperatures are believed to result from a high degree of crystallinity, which imparts structural stability, making the granules more resistant to gelatinization (Barichello et al., 1990; Hoover and Sosulski, 1985; Leszkowiat et al., 1990). High transition temperatures may also reflect more stable amorphous regions or a lower degree of chain branching (Biliaderis et al., 1980; Leszkowiat et al., 1990). Starch from Norchip had transition temperatures which differed most from the means; the onset and peak temperatures were each 3-standard deviations below the means. Genotypes W877 and ND651-9 showed the greatest differences between onset and peak temperature: 3.8°C vs a mean difference of 3.0°C for the 42 genotypes. The gelatinization enthalpy ranged from 16.7 J/g for 3 genotypes including Norchip, to 18.1 J/g for Gemchip and GS7232-4. A high gelatinization enthalpy also suggests a more stable granular structure due to greater crystallinity (Wong and Lelievre, 1982; Leszkowiat et al., 1990).

Published studies of DSC using potato starch reported both lower and higher transition temperatures. Onset and peak transition temperatures reported by Russell (1987) for a single potato starch sample were 4.0 and 3.3°C lower, respectively, than means in our study, but comparable to results reported here for Norchip. The gelatinization enthalpy in that study was 1.8 J/g lower (4.9 × S.D. lower) than our mean. Conversely, Barichello et al. (1990) reported onset and peak temperatures for Norchip and ND860-2 which were 5 to 7°C higher than our values for respective genotypes. Those workers used the same heating rate. Although water content was slightly different, transition tem-

peratures are not sensitive to water content in the range 50 to 90%. (Biliaderis et al., 1980; Shiotsubo and Takahashi, 1984; Barichello et al., 1990). Discrepancies in transition temperatures between the 3 studies may be partly related to use of different DSC instruments and procedures (personal communication, 1994, NC-136 Ad Hoc Committee on DSC) or to differing growing and storage conditions and starch isolation procedures. Barichello et al. (1990) suggested that the higher transition temperatures for ND860-2 compared to Norchip might be linked to resistance to chill-sweetening in that genotype. Transition temperatures for ND860-2 in our results were similar to most other varieties, whereas Norchip temperatures were lower (Table 1).

Other physical properties

Intrinsic viscosity is a measure of internal friction or resistance to displacement of high-polymeric molecules in solution (Leach, 1963). It is related to average molecular size of starch, chain rigidity, and branching or shape of the macromolecule (Launay et al., 1986; Baianu, 1992). In our tests, intrinsic viscosity [η] values of potato starches, ranged widely from 2.51 for Agassiz to 3.63 for GS7232-4, depending on potato genotype (Table 2). The lower values were similar to the 2.63 reported by Leach (1963) for unmodified commercial potato starch.

Water-binding capacity ranged from 77.2% for Tolaas to 89.6% for Snowden (Table 2). The ultra-structural (molecular arrangement, amorphous, and crystalline areas) and the compositional (mainly amylose, amylopectin and phosphorus) differences of the starches may affect availability of molecules to interact with water. For example, a loose association of amylose and amylopectin molecules in the native granules may contribute to a high water-binding capacity (Soni et al., 1987). Engagement of the hydroxyl groups to form hydrogen and covalent bonds between starch chains might lower water-binding capacity (Hoover and Sosulski, 1986). Therefore, differences among starches by potato genotype likely resulted from the different degrees of availability of water-binding sites, considered to be hydroxyl groups and inter-glucose oxygen atoms (Wootton and Bamunuarachchi, 1978).

Pasting properties, which indicate the degree of paste viscosity and stability of native potato starches were determined with a Brabender Visco-amylograph. Results from the Brabender Amylograph test (Table 3) included pasting temperature, peak temperature, peak viscosity, stability ratio, and setback ratio. Pasting temperature ranged from 62.3°C for Norchip to 68.5°C for Erik, similar to pasting temperatures (61.5 to 68.0°C) of Wiesenborn et al. (1994).

After the onset of pasting, the viscosity of most potato starches increased rapidly to peak viscosity. The highest peak viscosity was attained with E55-35 followed by Sebago and Superior. High viscosity is desirable for industrial uses in which a high thickening power is required. The amylogram of E55-35 is displayed beside that of Red Pontiac and ND651-9 (Fig. 1). The Red Pontiac amylogram was typical of potato starch amylograms; however, Red Pontiac has yielded atypically stable viscosities in some studies (McComber et al., 1988; Wiesenborn et al., 1994).

The extreme opposite to E55-35 was demonstrated in the amylogram for ND651-9, which did not exhibit a peak viscosity. The viscosity of the ND651-9 starch tended to increase continuously throughout the test. This viscosity pattern was classified as type C by Schoch and Maywald (1968). This is similar to the amylogram of a chemically cross-linked starch. Thus, ND651-9 starch might be used as a stabilizer or thickener in a canned product that requires retorting. Starch from ND651-9 also exhibited a high setback ratio, as did Mainechip.

Chemical properties

The amylose content of the 42 potato starch samples ranged from 22.6% for Krantz to 28.8% for Shasta (Table 2). Thus,

Table 2—Physicochemical properties of starch from 42 potato genotypes[a]

	Intrinsic Visc. [η]	WBC (%)	Amylose (%)	Phosphorus (ppm)
Agassiz	2.51	87.6 ± 0.00	24.9 ± 0.66	702 ± 0.0
Atlantic	3.01	86.2 ± 1.41	25.7 ± 0.32	908 ± 1.4
Bintji*	2.85	83.6 ± 0.00	25.6 ± 0.49	816 ± 2.5
C.M.C.	3.15	85.4 ± 0.28	25.5 ± 0.25	851 ± 2.8
Eide	3.15	88.8 ± 0.57	25.4 ± 0.38	888 ± 0.0
Erik	2.92	79.0 ± 0.85	24.3 ± 0.07	883 ± 0.0
Gemchip	3.21	83.4 ± 0.28	23.4 ± 0.83	816 ± 2.5
Irish Cobbler	3.32	85.2 ± 0.57	26.5 ± 0.18	626 ± 0.0
Kennebec	2.80	81.2 ± 0.00	23.2 ± 0.33	837 ± 0.0
Krantz	3.48	84.4 ± 0.57	22.6 ± 0.05	802 ± 0.0
Mainechip	3.09	88.4 ± 0.00	25.4 ± 0.57	675 ± 0.7
Monona	3.39	82.0 ± 1.13	26.3 ± 0.77	803 ± 1.2
Norchip*	3.24	89.2 ± 0.00	25.1 ± 0.24	685 ± 0.0
Norking Russ.	3.35	81.2 ± 0.00	25.1 ± 0.64	747 ± 2.5
Red Lasoda*	3.38	85.6 ± 0.00	23.3 ± 0.60	897 ± 4.9
Red Norland*	3.21	89.0 ± 0.85	24.1 ± 0.57	875 ± 19.3
Red Pontiac*	3.23	81.8 ± 0.28	27.0 ± 0.46	778 ± 0.0
Reddale*	3.23	86.4 ± 0.00	24.1 ± 0.45	951 ± 1.2
Redsen*	3.45	83.4 ± 0.28	24.6 ± 0.64	696 ± 1.3
Russet Burbank*	3.28	82.4 ± 0.00	23.7 ± 0.87	596 ± 3.8
Russet Norkota*	3.62	84.8 ± 0.57	25.6 ± 0.59	682 ± 0.0
Sebago	2.84	86.0 ± 0.57	25.8 ± 0.44	927 ± 6.4
Shasta	2.81	86.4 ± 0.28	28.8 ± 0.89	606 ± 4.9
Shepody	3.06	82.4 ± 0.00	27.6 ± 0.52	612 ± 0.0
Snowden	3.41	89.6 ± 0.57	24.3 ± 0.48	722 ± 0.0
Superior	3.25	87.0 ± 0.28	23.9 ± 0.26	940 ± 1.2
Tolaas	3.16	77.2 ± 0.57	23.4 ± 0.33	849 ± 1.3
Triumph*	3.12	79.2 ± 0.00	25.3 ± 0.49	837 ± 2.8
Waseca	2.94	82.8 ± 0.00	25.1 ± 0.43	862 ± 2.5
A80559-2	2.94	83.8 ± 0.28	26.0 ± 0.39	803 ± 2.8
C69-1A	3.09	86.2 ± 2.55	25.2 ± 0.39	974 ± 0.0
C71-18	3.14	83.2 ± 0.57	26.9 ± 1.23	858 ± 0.0
C71-41	3.25	79.4 ± 0.28	27.2 ± 0.51	857 ± 0.0
C72-93	2.89	88.4 ± 0.57	27.7 ± 0.47	716 ± 0.0
E55-35	2.89	89.0 ± 0.28	25.8 ± 0.76	1022 ± 0.0
E55-44	3.23	87.4 ± 0.28	25.2 ± 0.50	745 ± 0.0
GS7232-4	3.63	82.0 ± 0.00	27.5 ± 0.21	777 ± 0.0
ND651-9	3.39	85.8 ± 0.28	26.8 ± 0.78	599 ± 0.0
ND860-2	2.72	85.8 ± 0.28	26.2 ± 0.28	693 ± 0.0
ND1538-1 Russ	3.10	85.6 ± 0.00	24.5 ± 0.22	769 ± 0.0
W870	2.89	86.2 ± 0.28	23.7 ± 0.77	821 ± 1.2
W877	3.46	85.0 ± 0.28	27.4 ± 0.22	724 ± 0.0
Mean ± S.D.	3.15 ± 0.25	84.7 ± 3.05	25.4 ± 1.44	791 ± 108

[a] Values are average ± standard deviation. WBC = Water-binding capacity.

* Starch from these genotypes was also characterized for amylose and phosphorus content for the 1990 growing season (Wiesenborn et al, 1994).

significant differences occurred in amylose contents among potato genotypes. These were similar to previously reported results in which amylose contents of 44 samples representing 34 potato genotypes averaged 26.5 ± 1.4% using the same colorimetric method (Wiesenborn et al., 1994). However, those values were higher than those (21 to 22%) reported by others for potato starch (Swinkels, 1985; Zobel, 1988). The amylose contents of starch from genotypes Norchip, ND860-2, Atlantic, Russet Burbank, and Shepody were 1 to 7% higher than reported for those genotypes by Barichello et al. (1991). The different amylose values might be attributed to different growing conditions, starch isolation procedures and analytical methods.

Potato starch is remarkable food starches for its high level of phosphate esters. Phosphorus is an important factor in several functional properties. The phosphorus contents for the 42 potato starch genotypes ranged from 596 ppm to 1022 ppm (Table 2). The genotype E55-35 had higher phosphorus than did the other cultivars and selections. High phosphorus content in starch contributes to improved freeze-thaw stability, clarity, and viscosity of starch paste (Muhrbeck and Eliasson, 1991; Craig et al., 1989). Researchers have reported a similar range of phosphorus contents of potato starch from 500 to 1000 ppm (Biliaderis et al., 1980; Craig et al., 1989; Lim and Seib, 1993; Swinkels, 1985; Wiesenborn et al., 1994), except for Veselovsky's (1940) results which ranged from 630 to 1240 ppm for 69 potato genotypes.

Protein contents expressed as percentage of total dry weight were about 0.1% or less (data not shown). Thus, the purification procedure, which consisted of several repetitions of washing the

	Pasting temp. (°C)	Peak temp. (°C)	Peak visc. (B.U.)	Stability ratio	Setback ratio
Agassiz	66.3	92.0	935	0.46	1.12
Atlantic	65.7	83.0	1095	0.87	1.10
Bintji	65.4	80.7	1230	0.40	1.20
C.M.C.	65.5	90.2	1155	0.50	1.22
Eide	67.0	94.5	928	0.52	1.16
Erik	68.5	89.0	1195	0.50	1.18
Gemchip	66.5	81.7	1315	0.36	1.19
Irish Cobbler	65.8	92.6	1110	0.58	1.17
Kennebec	64.2	86.0	1235	0.40	1.15
Krantz	65.3	84.6	1245	0.40	1.16
Mainechip	64.5	94.3	1028	0.56	1.50
Monona	66.3	86.0	1213	0.40	1.14
Norchip*	62.3	90.7	970	0.41	1.17
Norking Russ.	66.0	93.0	1070	0.58	1.17
Red Lasoda*	65.9	88.3	1190	0.43	1.12
Red Norland*	66.6	95+2.2min[b]	985	0.58	1.10
Red Pontiac*	67.5	95+4min	980	0.66	1.07
Reddale*	64.5	77.4	1290	0.41	1.20
Redsen*	68.0	95+5.5min	755	0.78	1.11
Russet Burbank*	64.2	89.2	935	0.36	1.16
Russet Norkota*	65.4	93.9	1030	0.62	1.16
Sebago	67.0	78.7	1485	0.37	1.17
Shasta	67.0	95+1.2min	840	0.77	1.09
Shepody	66.7	95+4.5min	850	0.69	1.11
Snowden	63.9	84.7	1140	0.47	1.17
Superior	66.3	77.0	1455	0.37	1.16
Tolaas	64.0	81.5	1170	0.46	1.16
Triumph*	66.6	95+1.2min	1013	0.66	1.11
Waseca	67.9	95+4min	910	0.65	1.07
A80559-2	65.0	76.8	1310	0.36	1.14
C69-1A	64.7	84.7	1270	0.46	1.21
C71-18	65.2	89.0	1290	0.45	1.19
C71-41	66.2	92.7	1205	0.54	1.19
C72-93	65.8	95÷7min	790	0.88	1.15
E55-35	67.8	78.3	1585	0.46	1.08
E55-44	67.5	91.5	1078	0.56	1.11
GS7232-4	67.3	91.8	1130	0.50	1.16
ND651-9	66.9	N.P.	540	1.00	1.55
ND860-2	66.4	95+3min	898	0.59	1.13
ND1538-1 Russ	64.6	91.0	1070	0.54	1.16
W870	65.0	84.0	1170	0.47	1.16
W877	65.7	86.2	1130	0.50	1.22
Mean ± S.D.	65.9 ± 1.3		1100 ± 204	0.54 ± 0.1	1.17 ± 0.1

[a] Values are average ± standard deviation.
[b] Time at the peak during holding at 95° C.
B.U. = Brabender units
N.P. = No peak.
* Starch from these genotypes was also characterized for pasting properties for the 1990 growing season (Wiesenborn et al, 1994).

starch, successfully removed all but a small amount of protein. These low levels of protein should have no effect on functional characteristics.

Moisture contents ranged from 7.2% for Shasta to 16.79% for GS7232-4 (data not shown). The difference in range of moisture was probably due mainly to differences in ambient relative humidities and drying times.

Correlation of properties

Correlation coefficients were computed to examine relationships between measured thermal characteristics determined by DSC and other physical and chemical properties of the starch (Table 4). Transition temperatures (T_o, T_p, and T_c) of potato starch by DSC correlated positively with one another and with gelatinization enthalpy (ΔH) ($P < 0.01$, Fig. 2). These correlations were in agreement with previous reports for rice starches and foxtail millet starch (Russell and Juliano, 1983; Fujita et el, 1989). Fujita et al. (1992) reported a correlation between T_p and ΔH in crops with a waxy phenotype, such as rice, barley and proso millet, but it was not shown in a crop with no waxy phenotype, such as wheat. These correlations support the hypothesis that transition temperatures and ΔH are influenced by common factors such as degree of crystallinity.
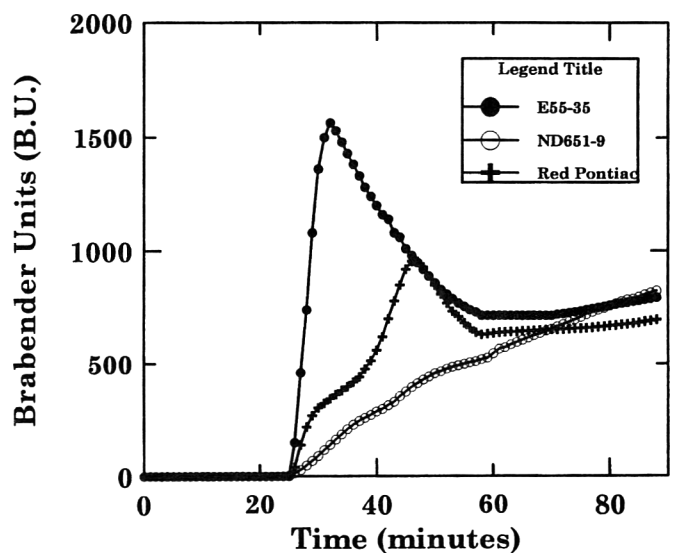

Fig. 1—Brabender amylogram for selected potato starches at 3.25% starch-water suspension.

The transition temperatures (T_o, T_p, and T_c) positively correlated with pasting temperature ($P < 0.01$) while gelatinization enthalpy (ΔH) positively correlated with pasting temperature ($P < 0.05$). T_o also correlated with phosphorus content and ΔH with peak Brabender viscosity ($P < 0.05$). Though statistically significant, the latter correlation was weak, as shown by the scatter plot (Fig. 3). These correlations suggest that DSC may be useful for screening potato genotype starches for high paste viscosity and high phosphorus content, which correlated with each other ($r = 0.72$, $P < 0.05$). For example, genotype E55-35 had the highest onset temperature, phosphorus content and viscosity.

Intrinsic viscosity and water-binding capacity did not correlate with other physical-chemical properties contrary to expectations. We hypothesized, for example, that intrinsic viscosity would be indicative of average molecular weight, and would correlate with granule crystallinity, such as gelatinization enthalpy, but no such correlation was observed. Starch intrinsic viscosity is probably also influenced by extent of amylopectin branching. The lack of correlation between water-binding capacity and amylose differed from Wootton and Bamunuarachchi (1978) who found that water-binding capacity of maize starch decreased with increasing amylose content. Hoover and Sosulski (1985) reported a positive correlation with amylose content. The amylose contents we found spanned a relatively narrow range.

Reproducibility and correlation of data from different years (1990 and 1991)

Unique, potato starch characteristics would have commercial value only if the desired characteristics could be reproduced consistently under reasonably acceptable growing conditions. Previously, starch from Red Pontiac tubers demonstrated interesting paste characteristics; however, that behavior was not consistently reproduced in subsequent years (Wiesenborn et al., 1994). Thus, properties of starch from 10 potato genotypes grown both in 1990 and 1991 were compared to study reproducibility of amylose, phosphorus, and paste characteristics (Table 5). The tubers were grown at the same location under conditions as similar as possible, but rainfall and temperatures varied naturally between the two seasons.

Half the potato starch genotypes (Bintji, R. Lasoda, R. Norland, R. Norkota, and Triumph) showed similar amylose contents (percent difference <3%) for the 2 years. Amylose content was only slightly related to genotype and environment.

For phosphorus. however, all genotypes except R. Norkota showed differences >3%. Some properties of starch have been

Table 4—Correlation coefficients for potato starch data (n = 42)

	DSC				Intrinsic Viscosity	Water-binding capacity
	To[a]	Tp[b]	Tc[c]	ΔH[d]		
DSC						
To	1.00					
Tp	0.97**					
Tc	0.71**	0.84**				
ΔH	0.53**	0.52**	0.41**			
Intrinsic viscosity	−0.10	−0.07	−0.02	0.13		
Water-binding capacity	−0.16	−0.08	0.11	−0.04	−0.12	
Amylose	0.14	0.21	0.15	−0.15	−0.06	0.02
Phosphorus	0.36*	0.30	0.30	0.25	−0.16	−0.03
Amylograph						
Paste temp.	0.81**	0.85**	0.70**	0.36*	0.07	−0.15
Peak visc.	0.27	0.19	0.17	0.34*	−0.06	−0.05
Stability ratio	0.04	0.09	0.02	−0.25	0.02	0.06
Setback ratio	−0.21	−0.13	−0.09	−0.21	0.18	0.12

[a] Onset temperature by DSC.
[b] Peak temperature by DSC.
[c] Conclusion temperature by DSC.
[d] Gelatinization enthalpy by DSC.
* and ** Significant at $P < 0.05$ and $P < 0.01$ levels of probability, respectively.
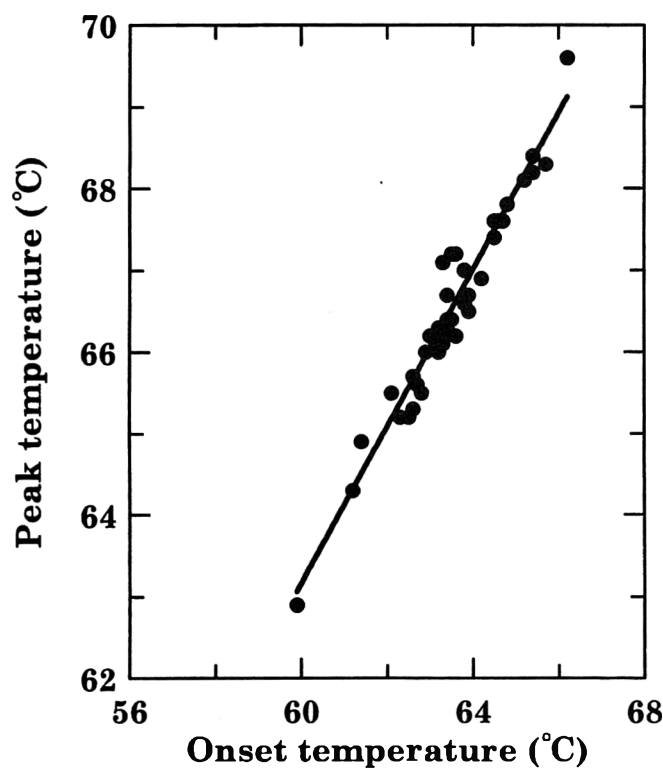
Fig. 2—Scatter plot showing relationships between onset temperature and peak temperature by DSC (n=42, r=0.97).
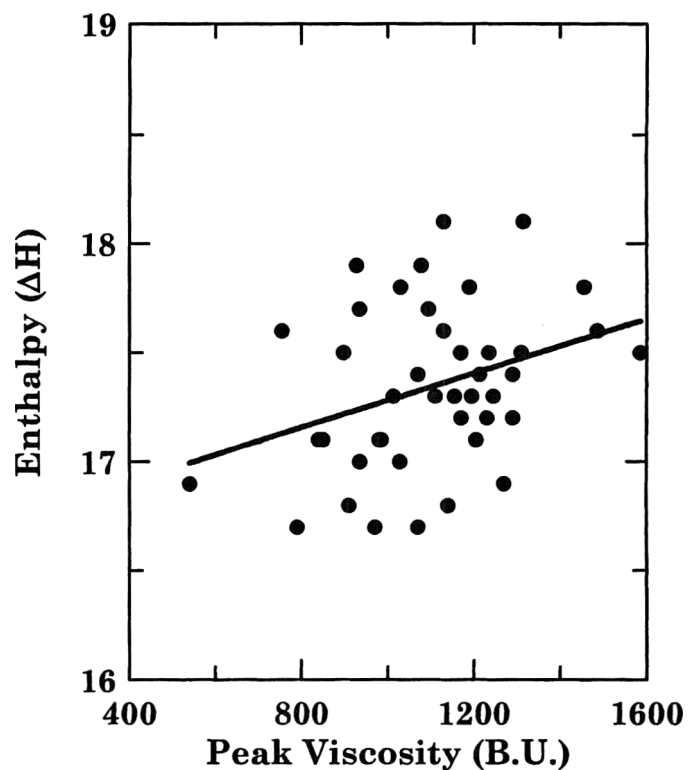
Fig. 3—Scatter plot showing relationships between gelatinization enthalpy (ΔH) by DSC and peak Brabender viscosity (n=42, r=0.34).

influenced by genetic and environmental factors during plant development (Asaoka et al., 1984, 1991, 1992; Gudmundsson and Eliasson, 1991). This appeared to be especially true of phosphorus content in potato starch. The pasting temperature was reproduced to ± 1°C in 7 of the 10 genotypes. Other paste characteristics showed more variability, probably due in part to variability in phosphorus content (Wiesenborn et al., 1994).

Environmental factors appeared to affect phosphorus content and other potato starch properties. We expected that genotypes grown at the same time and in close proximity might show similar changes over the two years. A least squares fit showed that pasting temperature data from 1991 correlated with data from 1990 (P < 0.01, r = 0.83), as did phosphorus content data (P < 0.01, r = 0.80). Genotypes R. Lasoda and Reddale contained the highest phosphorus and R. Burbank the lowest phosphorus in both years. Stability ratios correlated (P < 0.05, r = 0.66); however, correlations for peak viscosity (r = 0.34) and setback ratio (r = −0.49) were not significant (P > 0.05).

CONCLUSIONS

POTATO STARCH SAMPLES from various genotypes show differing thermal properties as determined by DSC. Such properties correlated with other physical-chemical characteristics of the starch. Given the small sample size and simple preparation methods, DSC may be useful as a screening tool for potato starch samples such as tailoring starch properties through genetic engineering. The DSC data did not account for the wide range of potato starch pasting characteristics. Other physical and chemical data are needed to explain this range. Some genotypes namely E55-35, and ND651-9, demonstrated unique paste characteristics which could be of interest in certain food applications.

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Table 5—Starch characteristics of potato genotypes from 1990 and 1991

	Amylose (%)	Phosphorus (ppm)	Pasting temp. (°C)	Peak at temp. (°C)	Peak viscosity (B.U.)	Stability ratio	Setback ratio
Bintji (90)	25.9	816	63.6	91.7	1000	0.52	1.09
Bintji (91)	25.6	745	65.4	80.7	1230	0.40	1.20
Norchip (90)	27.4	738	63.0	95.0	1050	0.62	1.04
Norchip (91)	25.1	685	62.3	90.7	970	0.41	1.17
R. Lasoda (90)	23.5	925	65.9	95+2.0 min[a]	1110	0.78	N.A.
R. Lasoda (91)	23.3	897	65.9	88.3	1190	0.43	1.12
R. Norland (90)	24.5	797	65.1	95+11.5 min	630	0.99	1.23
R. Norland (91)	24.1	875	66.6	95+2.2 min	985	0.58	1.10
R. Pontiac (90)	29.2	672	65.5	95+9.3 min	745	0.95	1.16
R. Pontiac (91)	27.0	778	67.5	95+4.0 min	980	0.66	1.07
Reddale (90)	30.0	848	64.0	94.1	960	0.63	1.13
Reddale (91)	24.1	951	64.5	77.4	1290	0.41	1.20
Redsen (90)	26.5	738	68.0	95+3.2 min	930	0.77	1.05
Redsen (91)	24.6	696	68.0	95+5.5 min	755	0.78	1.11
R. Burbank (90)	26.1	644	62.8	95+1.3 min	920	0.64	1.09
R. Burbank (91)	23.7	596	64.2	89.2	935	0.36	1.16
R. Norkota (90)	25.8	693	65.3	95+0.3 min	870	0.71	1.10
R. Norkota (91)	25.6	682	65.4	93.9	1030	0.62	1.16
Triumph (90)	26.0	744	67.2	N.P.	N.P.	1.00	1.16
Triumph (91)	25.3	837	66.6	95+1.2 min	1013	0.66	1.11

N.A.: Not available.
N.P. = No peak.
Source of the 1990 results: Wiesenborn et al. (1994).
[a] Time at peak during holding at 95 °C.

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Ms received 12/19/94; revised 4/10/95; accepted 4/19/95.

Protein Concentrates from Unstabilized and Stabilized Rice Bran: Preparation and Properties

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

Protein concentrates were prepared from commercially available unstabilized and heat stabilized rice bran by alkaline extraction and isoelectric precipitation. Stabilized rice bran had lower protein extractability at all particle sizes (75μ to 150μ). Both unstabilized rice bran protein concentrate (URBPC) and stabilized rice bran protein concentrate (SRBPC) showed maximum nitrogen solubility at pH 8.0. URBPC (71.5% protein) and SRBPC (50.9% protein) showed differences in amino acid contents. SDS-PAGE of protein concentrates revealed several common components and absence of certain components from SRBPC. Protein denaturation due to commercial heat stabilization impaired extractability of proteins and influenced its quality.

Key Words: rice bran, protein, concentrates, amino acid, stabilization

INTRODUCTION

RICE BRAN is an under-utilized milling by-product of rough rice. In 1994, about 155 million cwt of rice was produced in the U.S., resulting in about 12.4 million cwt of bran (Arkansas Agricultural Statistics, 1993). The rice processing industry has considered the commercial potential of this material. To enhance keeping quality, rice bran is stabilized by heat treatment for 1 to 20 min. depending on method of stabilization, to inactivate lipases (Sayre et al. 1982). Stabilized rice bran is marketed for food and feed uses (Hargrove, 1994). The healthful hypolipidemic and hypocholesterolemic effects of rice bran have been well documented (Suzuki, 1982; Seetharamaiah and Chandrasekhar, 1988; Kahlon et al., 1990). Rice bran is also considered as a source of hypoallergenic proteins and may serve as a suitable ingredient for weaning formulations (Burks and Helm, 1994). Rice bran is a rich source of dietary fiber, and is also rich in proteins with high nutritional value (Saunders, 1990). Several studies were conducted on preparation of protein concentrates and isolates from rice bran (Lynn, 1969; Chen and Houston, 1970; Lew et al., 1975; Maki and Tashiro, 1983; Saunders, 1990; Landers, 1992). However, limited information is available on extraction, characterization and properties of protein concentrates from unstabilized or stabilized rice bran products. Our objective was to prepare protein concentrates from commercial unstabilized and stabilized rice bran and compare their properties.

MATERIALS & METHODS

UNSTABILIZED RICE BRAN was obtained from Riceland Food Inc. (Stuttgart, AR. 72160) and stabilized rice bran from Riviana Food Inc. (Abbeville, LA 70511) packaged in 2.27 kg polyethylene bags and stored at −5°C.

Defatting

Rice bran samples were dispersed (1:4) in technical grade hexane (Fisher Scientific, USA), defatted at setting 40 (T-Line lab stirrer, Talboys Engineering Corp., Emerson, N.J.) for 30 min., and centrifuged (IEC, CRU-5000) at 2500 × g for 10 min. at room temperature (≈23°C).

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Defatted rice bran sample + distilled deionized water (1:4 to 1:6) pH adjusted to 9.5 stirred 30 min. (room temperature)

↓

Centrifuged 10,000 X g for 30 min. (room temperature)

↓

Decant, pH of the supernatant adjusted to 4.5, centrifuged 10,000 X g for 30 min.(room temperature)

↓

Precipitate washed (pH 4.5), suspended in distilled deionized water (pH 7.0)

↓

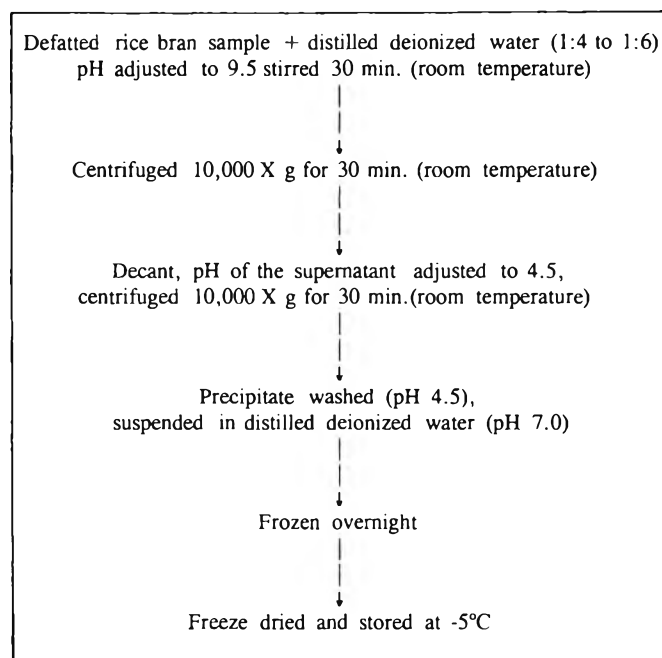Frozen overnight

↓

Freeze dried and stored at -5°C

Fig. 1—Flow diagram of preparation of protein concentrates.

The sediment was extracted once again following the same procedure. Defatted rice bran samples were air dried overnight under a hood.

Milling and fractionation

Defatted rice bran samples were milled once in a Cyclotec mill (Cyclotec 1093 Sample Mill, Tecator AB Box 70, Higanas, Sweden) and sieved through mesh sizes 200 (75μ), 170 (90μ), 140 (106μ), 120 (125μ), and 100 (150μ) (U.S. Standard Testing Sieve, ASTM E-11 Specification) for 3 min. Individual fractions from various mesh screens were packaged in polyethylene bags, labelled and stored at −5°C.

Proximate analysis

Percent moisture, crude fat, protein, and ash contents of defatted unstabilized and stabilized rice bran samples were determined according to AOAC procedures (1984).

Preparation of protein concentrates

Alkaline extraction procedures followed by isoelectric precipitation were used to prepare protein concentrates (Fig. 1). Defatted rice bran samples of varying particle sizes were dispersed in distilled deionized water. A sample to water ratio of 1:4 was used for unsieved, 200 and 170 mesh samples and a ratio of 1:5 was used for 140 and 120 mesh samples. For samples < 120 mesh, a ratio of 1:6 was used.

Rice bran samples were dispersed in water at appropriate dilutions and the pH was adjusted and maintained at 9.5 for 30 min stirring. After 30 min the product was centrifuged at 10,000 × g for 30 min at ambient temperature to remove insoluble material. After centrifugation, the supernatant was adjusted to pH 4.5 with 1.0N HCl to precipitate proteins and centrifuged again at 10,000 × g for 30 min. at ambient temperature. The precipitate was washed with distilled deionized water (pH 4.5) and

Table 1—Proximate composition of defatted unstabilized and stabilized rice bran

Sample	Moisture (%)	Crude fat (%)	Protein (%)	Ash (%)
UDRB[c]	10.05[a]	2.63	15.32[a]	11.87
SDRB[c]	7.67[b]	2.74	17.72[b]	12.74

[ab] Mean values in the same column with different superscripts are significantly different (P < 0.05).

[c] UDRB and SDRB are unstabilized defatted rice bran and stabilized defatted rice bran respectively.

Table 2—Milling recovery[d] and protein content of rice bran protein concentrates

Mesh size	Unstabilized		Stabilized	
	Recovery, %	Protein, %	Recovery, %	Protein, %
Unsieved	72.4	71.5[a]	72.2	50.9[a]
200	40.1	63.3[b]	40.2	50.7[a]
170	3.7	68.0[a]	4.9	47.3[b]
140	3.7	70.7[a]	4.9	43.7[b]
120	4.9	70.9[a]	7.7	43.1[b]
100	7.9	67.7[a]	4.7	38.9[c]
< 100	11.6	58.4[c]	10.5	33.5[c]

[abc] Mean values in the same column with different superscripts are significantly different (P < 0.05).

[d] Milling recovery calculated based on weight of the defatted material.

dispersed in a small amount of distilled deionized water (pH 7.0). The dispersed product was freeze-dried and stored at −5°C.

Nitrogen solubility

Nitrogen solubility of the protein concentrates was determined by using a modification of the procedure of Betschart (1974). Protein concentrate solutions (1%) were made by weighing equivalent amounts of samples directly into 50 mL centrifuge tubes. Two tubes for each replication were prepared and adjusted to 6 different pH levels (2.00, 4.00, 6.00, 8.00, 10.00, and 12.00) using 1.0, 0.1, or 0.01N NaOH or HCl. The suspensions were shaken (Lab-Line Environ-Shaker, Lab-Line Instrument, Inc. Melrose Park, IL) at 250 rpm for 30 min and centrifuged at 5,000 × g for 15 min at ambient temperature. The supernatants were filtered (Whatman No. 4) and analyzed for nitrogen (AOAC, 1984), and nitrogen solubility was calculated:

$$\text{Nitrogen solubility, \%} = \frac{\text{Nitrogen in supernatant}}{\text{Nitrogen in 100 g of sample}} \times 100$$

Amino acid analyses

Amino acid compositions of unstabilized and stabilized protein concentrates from unsieved fractions were determined using an amino acid analyzer (LKB Biochrom Model 4400, LKB Biochrom Ltd., Science Park, Cambridge, England). Samples were hydrolyzed in 6N HCl at 128°C for 15 hr. A cation-exchange column was used for amino acid separation. A photometer output linked to a recorder (LKB 2220, Recording Integrator) was used to record total retention times for individual amino acids. The area under each peak was used to quantitate individual amino acids in the original mixture.

Electrophoresis

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) of the samples was performed according to Laemmli (1970). A Bio-Rad vertical slab gel electrophoresis unit model Protean™ and power supply model 3000 Xi (Bio-Rad Laboratories, Richmond, CA) were used. The stacking gel and separating gel were 8% and 12% polyacrylamide respectively. Gels were scanned using a densitometer (Bio-Rad Model GS-670 Imaging densitometer).

Statistical analyses

Four replications of the fractionation and extractions were performed. A completely randomized design was used. Least square means procedures were used to separate means and differences reported were significant at P < 0.05 (SAS Institute, Inc., 1984).

RESULTS & DISCUSSION

Proximate composition

Proximate composition of commercially available defatted unstabilized and stabilized rice bran samples showed differences in protein and moisture (Table 1). No differences were observed between defatted samples in fat content. Defatting samples twice with hexane did not result in complete removal of fat. The protein contents of both unstabilized and stabilized rice bran were within the ranges reported (Luh, 1980).

Protein concentrates

Milling recovery and corresponding protein content of unstabilized and stabilized rice bran protein concentrates were compared (Table 2). Both samples showed a similar particle size distribution. A single grinding step resulted in much higher content of 200 mesh fractions. Protein content of the concentrates prepared from different fractions showed differences (Table 2). Concentrates prepared from 200 mesh unstabilized rice bran had lower protein contents than those prepared from other fractions. Type a and b particles of rice bran as categorized by Pineda (1976) with a particle size of 75μ might be a major contaminant in 200 mesh fractions. These particles include several non-protein components such as cellulose, hemicellulose, pentosans, and lignin. Protein extracted did not change as particle size decreased from 170 mesh to 100 mesh. However, when the particle size was < 100 mesh, there was a significant decrease in protein extracted (P < 0.05). As particle size increased, the sample became less floury, more water binding and hence required a higher effective volume of extractant. Our pH of 9.5 was not effective in extracting protein from this fraction.

A significant reduction in percent protein extracted was observed in all fractions of stabilized rice bran when compared to unstabilized rice bran. As particle size increased, the extraction procedure became less effective. Protein concentrates from fractions of < 100 mesh had the lowest protein content (33.5%). Three different processes of heat stabilization of rice bran have been reported: retained moisture heating, added moisture heating, and dry heating at atmospheric pressure (Sayre et al., 1982). Extrusion cooking, a type of retained moisture heating, as used in the production of the stabilized rice bran we used, involves heating at 125–135°C. High temperatures involved in stabilization processes may affect protein solubility. Prakash and Ramanathan (1994) reported a decrease in protein extractability due to heat stabilization. Protein solubility and extractability has been reported in the order: untreated > acid stabilized > parboiled > heat stabilized.

Reductions in particle size have been reported to increase extractability of proteins from rice bran (Betschart et al., 1977). Milling and sieving resulted in reduction of particle size. However, during milling, all components of rice bran such as cellulose, hemicellulose, lignin and pentosans, are reduced to different particle sizes. Thus, besides proteins, other components of rice bran are also present in samples from every mesh size. Grinding of the sample after air classification was reported to increase the protein content of rice bran fractions. But, the fiber content of such fractions may be high (Houston and Mohammad, 1966).

Protein concentrates prepared from unsieved fractions did not show any differences in protein content than the fractions with highest protein content. The milling yield of unsieved fraction was about 72%, higher than the yield of other particle sizes (Table 2). Protein content of unsieved, unstabilized rice bran protein concentrate (URBPC) and stabilized rice bran protein concentrate (SRBPC) were 71.5% and 50.9% respectively. This was in agreement with values for protein content of similar samples reported by Prakash and Ramanathan (1994) who used a higher pH (pH 11.0) for protein extraction. The protein contents of the URBPC we obtained were about 15% higher than those reported for concentrates obtained by alkaline extraction at pH
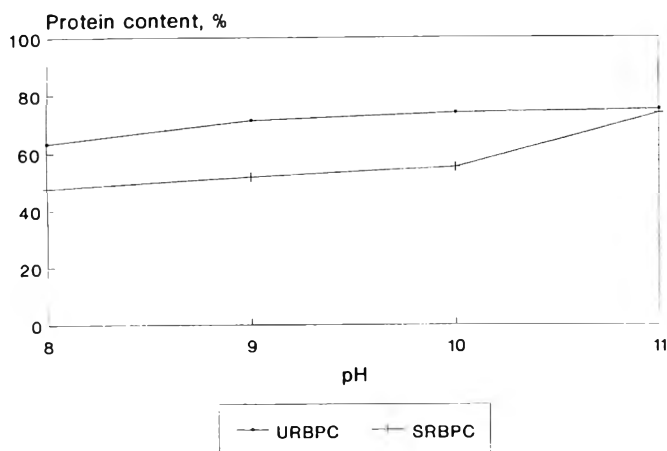
Fig. 2—Protein content of concentrates as affected by pH of extraction. URBPC = unstabilized rice bran protein concentrate, SRBPC = stabilized rice bran protein concentrate.
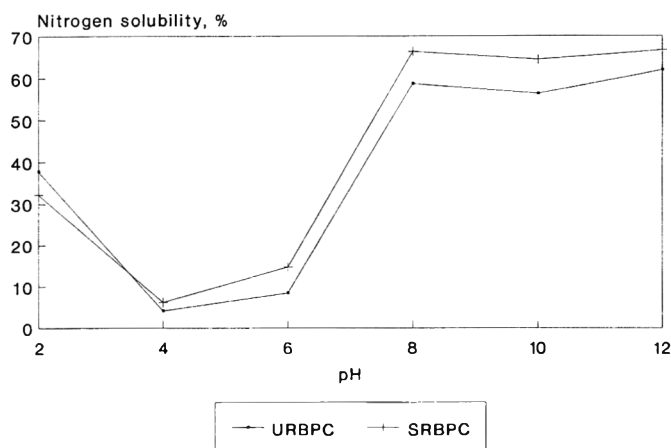

Fig. 3—Nitrogen solubility of rice bran protein concentrates at varying pH. URBPC = unstabilized rice bran protein concentrate, SRBPC = stabilized rice bran protein concentrate.

9.0 (Chen and Houston, 1970; Bera and Mukherjee, 1988; Landers, 1992).

pH 9.5 vs higher pH

Chen and Houston (1970) reported that the percent of protein extracted showed a linear increase from pH 7.5 to pH 11.0, and then decreased at pH 12.0 due to solubilization of nonprotein solids. Lew et al. (1975) prepared protein concentrate from rice bran by alkaline extraction at pH 11.0. It has also been reported that purity of proteins decreased when extracted at pH 12.0 (Juliano, 1985). Besides resulting in a severe protein degradation, alkaline pH may cause several changes in amino acids resulting in formation of lysinoalanine, lanthionine, and hence a decrease in nutritive value (DeGroot and Slump, 1969). A combination of high alkaline pH and high temperature resulted in protein degradation and undesirable flavor effects in rice bran protein concentrate (Lynn, 1969). Protein content of the concentrates showed a steady increase as pH of extraction increased from 8.0 to 11.0 (P < 0.05) (Fig. 2). A significant increase in protein extractability of stabilized rice bran was observed at pH 11.0 compared to pH 10.0. It is possible to maximize extractability of proteins from rice bran using a higher extraction pH. However it is important to retain the desirable attributes of the proteins, especially nutraceutical properties. The pH of extraction should be of prime importance in view of its undesirable

Table 3—Amino acid composition of rice bran protein concentrates[c]

Amino acid[d]	URBPC	SRBPC
Asp	10.57[a]	11.50[a]
Thr	3.86[a]	3.471[a]
Ser	4.58[a]	3.47[b]
Glu	11.91[a]	26.62[b]
Gly	6.89[a]	9.40[b]
Ala	7.86[a]	6.44[b]
Val	7.72[a]	5.27[b]
Leu	9.52[a]	6.00[b]
Tyr	3.86[a]	3.11[a]
Phe	5.89[a]	2.47[b]
His	4.96[a]	8.80[b]
Lys	7.08[a]	6.27[b]
Arg	13.65[a]	16.20[b]

[a,b] Mean values in the same row with different superscripts are significantly different (P < 0.05).
[c] Mean of three replications.
[d] Amino acid mg/100 mg of protein.

effects on native state of proteins. This is important for their antigenic properties.

Nitrogen solubility

Nitrogen solubility of proteins has widely been used to express and predict the solubilities of protein preparations. Nitrogen solubilities of URBPC and SRBPC were compared (Fig. 3). Over the pH range (2.00–12.00), both samples showed the lowest solubility at pH 4.0. Below and above this pH they showed a gradual increase in solubility. At pH 2.0 the samples had about 40% solubility. Maximum solubilities for both samples were observed at pH 8.0, beyond which there was not much increase. Champagne et al. (1985) reported a possible interaction of copper and zinc with proteins at alkaline pH. Such an association could adversely affect protein solubility. Extraction procedures could be another factor affecting solubility of protein preparations. Albumins and globulins were reported to be the major proteins of rice bran. The mean ratio of albumin:globulin:prolamin:glutelin in rice bran were reported as 37:36:5:22 (Cagampang et al., 1966). Since globulins are only about 1/3 of rice bran proteins, neutral salts such as sodium chloride may not have a significant effect on solubility of rice bran proteins. However, addition of sodium chloride and sodium dodecyl sulfate increased extractability of proteins in the pH range 2.00–6.00 (Prakash and Ramanathan, 1994). In our results, although SRBPC samples showed much lower extractability, the nitrogen solubility of the protein concentrates were similar to that of URBPC. Stabilized rice bran had much lower protein solubility than untreated (Prakash and Ramanathan, 1994). However, those samples were extracted at pH 11.0. The lower pH of extraction we employed would have a relatively milder effect on protein denaturation and hence might increase solubility.

Amino acid content

Out of the 13 amino acids tested, the contents of 10 were different between stabilized and unstabilized bran (Table 3). Both samples had a high content of lysine, the first limiting amino acid in cereal proteins. No differences were found in threonine, tyrosine and aspartic acid contents. SRBPC samples had higher glutamic acid, glycine, arginine and histidine, while URBPC samples had higher serine, alanine, valine, leucine, phenylalanine, and lysine. Acid precipitated rice bran protein concentrates reportedly had higher protein efficiency ratio (PER) values (1.99) that were better than proteins of soybeans (0.7–1.8), corn (1.2) and wheat (1.0) (Liener, 1972). Maki and Tashiro (1983) reported that rice bran protein concentrate was rich in good quality protein and comparable to casein in terms of essential amino acids except tryptophan. The amino acid value for rice bran protein concentrate was reported to be 80, while casein had a value of 85 (FAO/WHO, 1973). Differences in individual amino acid contents between unstabilized and par-
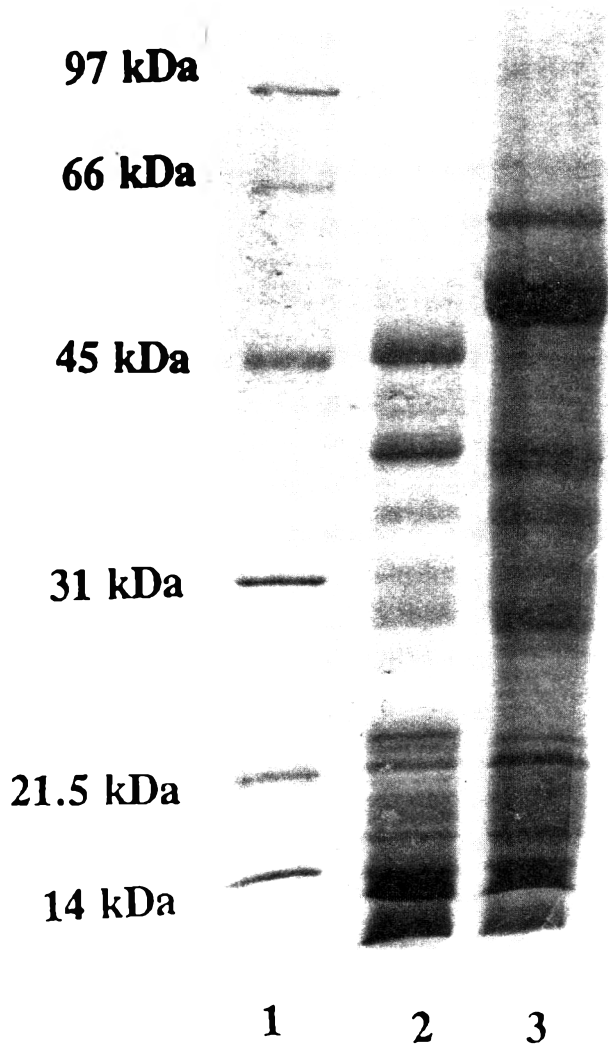
Fig. 4—SDS-PAGE of protein concentrates. Lane 1 = URBPC, Lane 2 = SRBPC, Lane 3 = MW Standards (BIO-RAD).

boiled rice bran have been generally reported around 10% (Luh, 1980). About 16% of the nitrogen in rice bran has been attributed to non-protein nitrogen (Baldi et al., 1976). The difference between protein content and total amino acid recovered corresponds to the reported non-protein nitrogen content. Acid hydrolysis at higher temperatures was not suitable to quantitate cystine, methionine, and proline. Reported data on amino acid composition of rice bran show wide variations. Apart from analytical procedures employed, rice variety and milling type seem to be sources of variations (Ronda and Soto, 1965).

The amino acid content and distribution of protein concentrates we found were similar to reported data for rice bran protein concentrates prepared at pH 9.0 (Bera and Mukherjee, 1988). However, the amount of protein extracted was about 20% more in our results.

SDS-PAGE

Electrophoresis of both protein concentrate samples revealed several components and differences in distribution pattern (Fig. 4). SRBPC was distinctly different from URBPC as evident from the absence of several components in the MW range 45 kDa to 97 kDa. Densitometry also revealed several low molecular weight fractions in URBPC in the range of 13 kDa to 26 kDa, most of which were absent in SRBPC. Protein denaturation

during stabilization might account for the absence of several of these components. The SRBPC sample revealed the presence of additional components in the MW range 21 kDa to 25 kDa, perhaps due to heat induced polymerization of proteins as a result of stabilization. Protein-polysaccharide interactions have been reported to occur in complex food systems (Ledward, 1994). During denaturation, unfolding of protein exposes charged sites that might interact with oppositely charged species from non-protein components. Such interactions might be responsible for altered protein extractions and precipitation. Lower extractability of proteins from stabilized rice bran under our conditions might be another reason for absence of certain components.

CONCLUSIONS

SIGNIFICANT DIFFERENCES were found in protein concentrates from unstabilized and stabilized rice bran. Although the heat stabilization process inactivates lipases and extends keeping quality of rice bran, it results in an irreversible effect on quantitative as well as cualitative protein recovery. Such samples had a decreased protein extractability, differences in amino acid content and electrophoretic patterns. Methods of rice bran stabilization are an important factor to consider before producing protein ingredients from rice bran. Protein extraction procedures should be carefully controlled to produce protein concentrates with desirable functional properties. A pH of 9.5 might be considered moderate but useful to prepare rice bran concentrates with > 70% protein. Extracting rice bran proteins in their least denatured state may be necessary to preserve nutritional and functional properties. Mild extraction conditions are important for producing protein concentrates and isolates from rice bran.

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β-Glucan Enrichment of Barley Fractions by Air Classification and Sieving

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ABSTRACT

Barley (Steptoe, Crystal and/or Waxbar) was dry milled and fractionated by small scale air classifiers and batch or continuous feed sifters to produce β-glucan-rich fractions. Sieving of barley (all cultivars) yielded fines fractions containing 88–90% starch and a final coarse enriched fraction containing 40–45% total dietary fiber including 16–19% β-glucans and up to 82 μg/g total tocols. The enriched fraction was 20–25% (by wt) of the dehulled barley. The highest β-glucan content by air classification was 9% due to ineffective separation of fines (particles <45 μm). Dry fractionation of barley produced a value added enriched product enhanced in factors (ie. tocols, dietary fiber, β-glucans) which slow oxidation, lower cholesterol, moderate glycemic response and which provide unique functional properties in food systems.

Key Words: barley, beta glucan, enrichment, air classification, dry milling

INTRODUCTION

BARLEY is high in dietary fiber which includes β-glucans. Dietary fiber, consisting of plant cell wall remnants including all polysaccharides and lignin that are resistant to endogenous secretions of the human digestive tract, have been related to prevention and treatment of coronary heart disease and diabetes. Reduced risk of coronary heart disease is associated with lowering of serum cholesterol and prevention/control of diabetes is associated with moderation of glycemic and insulin response. Studies of dietary fiber indicate that soluble fibers such as pectin, gums, and β-glucans (mixed linked (1-3),(1-4)-β-D-glucan) lower serum cholesterol (Anderson and Chen, 1979; Chen et al., 1981; Fadel et al., 1987; Klopfenstein and Hoseney, 1987; Newman et al., 1989a; Wood et al., 1989a; Newman et al., 1992; Kahlon et al., 1993) and moderate glycemic and insulin response (Jenkins et al., 1977;1980; Kiehm et al., 1976; Anderson and Ward, 1979; Potter et al., 1981; Yokoyama et al., 1993). Alpha-tocotrienol and cereal fractions containing it have been reported to lower serum cholesterol by inhibition of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA), the first rate limiting enzyme in cholesterol biosynthesis (Qureshi et al., 1985, 1986, 1991). The α-tocotrienol, α-tocopherol (vitamin E), other tocopherols (T), and tocotrienols (T3) contribute effective antioxidant activity (Gapor et al., 1989) which may protect unsaturated lipids in membrane tissue against peroxidation (Scott, 1978; Burton and Traber, 1990).

Incorporation of milled whole cereal into food systems (ie. breads, pasta, etc) is limited due to undesirable effects on color, texture, and/or loaf volume. Use of enriched fiber and β-glucan fractions from barley have enabled preparation of acceptable muffins, breads, and pasta (Hudson et al. 1992; Knuckles et al., 1992a) with significantly increased dietary fiber and β-glucan content.

Dry fractionation of cereals to produce fractions enriched in fiber and β-glucans may provide an economic advantage over methods utilizing solvents to produce β-glucan-rich fractions from oats (Inglett, 1993; Wood et al., 1989b). Utilization of

solvents requires the additional cost of solvent removal and/or recovery. Barley fractions produced by grinding with roller mills or hammer mills and particle fractionation by screening or air classification yielded fractions enriched in β-glucan and tocols (Bach Knudsen and Eggum, 1984; Danielson et al., 1994; Foehse, 1991; Bhatty, 1992; Wang et al., 1993). Generally, β-glucan concentrations of these fractions were <16%. Laboratory studies at the USDA's Western Regional Research Center demonstrated that barley fractions containing 19% β-glucan could be produced (Knuckles et al., 1992b).

Our objective was to carry out pilot scale fractionation of barley to produce a β-glucan-enriched fraction for product development (bread, muffins, and pasta) and studies of glycemic properties and cholesterol levels in animals, and humans. Air classification, batch, and continuous sifters were compared as methods for fractionation.

MATERIALS & METHODS

Barley

The barleys were Steptoe (ARCO registered seed, 1989, Trost Feed & Seed Co., St. Anthony, ID), Crystal (pearled, 1991, Wallace Grain & Pea, Palouse, WA) and Waxbar (cleaned, 1991, Western Plant Breeders, Bozeman, MT). Waxbar is a "waxy hull-less" type barley, whereas, Steptoe and Crystal are normal starch, covered type barleys. Previous work has shown waxy types to be higher in β-glucans than normal starch barleys (Xue et al., 1991).

Process equipment and descriptions

Preparations of β-glucan-rich fractions required multiple milling and separation steps. Crystal, Steptoe, and Waxbar barley were ground and sieved (325 mesh) in three successive steps where the coarse fractions of preceding steps were reground and resieved. Fractions A, B, and C were the fines (<325 mesh) from the three processing steps, respectively. Fraction D was the coarse (>325 mesh) fraction from the third processing step. The fines fractions A, B, and C would likely be combined in a production process although they were kept and analyzed separately to aid in monitoring and evaluating the β-glucan enrichment process. Crystal barley was used when some individual pieces of equipment were evaluated on a one pass basis.

Milling. Steptoe barley (230 kg) was dehulled at 230 g/min in a one-pass rice whitening machine (Satake Engineering Ltd., Tokyo, Japan) before milling. Crystal and Waxbar barleys were milled as received. A small-scale hammer mill (PMCO, model 2 DH, Hosokawa Micron, Summit, NJ) fitted with 0.254, 0.686 or 1.18 mm screens was used to prepare milled Crystal barley flours #1–3, respectively. Feed rate to the mill was ~136 kg/hr. A hammer mill (Pulvacron, Bepex PC-20, Hosokawa Micron, Summit, NJ) was used to prepare Crystal barley flour #4. The feed rate to the mill was ~365 kg/hr. The hammer mills were not available for sieving studies. A pin mill (Alpine, model 160 Z, Hosokawa Micron International, Inc., Summit, NJ) was used to prepare Crystal barley flour #5, Steptoe, and Waxbar flours for sieving studies. Feed rate to the pin mill was 16.2 kg/hr, measured for 5 kg of feed several times during processing.

Air classification. Crystal barley flours #1–3 and remilled coarse fractions from flour #3 were separated into coarse and fine fractions by small-scale air classification (Raymond air-classifier, ~20 cm model, Raymond Pulverizer Div., A A B Raymond, Niles, IL). The weights of coarse and fine fractions were recorded. Crystal barley flour #4 (900 kg) was fractionated in a large-scale air classifier (Alpine, model MS-2, Hosokawa Micron, Summit, NJ). Only weight, particle size distributions and β-glucan analyses were made on air classification fractions since β-glucan enrichment was low.

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Table 1—Percent coarse particles (> 45μm, < 300 μm) in milled barley fractions

Barley/Fraction[a]	Mill[b]	Screen size mm	Coarse particles %
Crystal			
Pearled (#1)	DH2	0.254	34.2
Pearled (#2)	DH2	0.686	43.0
Pearled (#3)	DH2	1.180	57.4
Pearled (#4)	Pulvacron	—	56.6
Pearled (#5)	Pin mill	—	45.5
1st Coarse fraction	DH2	1.180	73.1
1st Coarse fraction	Pin mill	—	72.7
2nd Coarse fraction	DH2	1.180	81.0
2nd Coarse fraction	Pin mill	—	81.3
Steptoe			
Dehulled	Pin mill	—	47.2
1st Coarse fraction	Pin mill	—	65.3
2nd Coarse fraction	Pin mill	—	78.4
Waxbar			
Cleaned	Pin mill	—	48.7
1st Coarse fraction	Pin mill	—	68.6
2nd Coarse fraction	Pin mill	—	82.0

[a] Numbers in parentheses are flour identifications given in the text.
[b] DH2 and Pulvacron are small and large scale hammer mills, respectively.

Table 2—Yield of barley fractions[a]

Fraction	Steptoe	Crystal	Waxbar
Milled barley[c]	100.0	100.0	100.0
A	53.6[a] ± 2.7	56.5[a] ± 2.4	22.9[b] ± 1.7
B	16.7[b] ± 0.7	14.8[b] ± 1.6	38.3[a] ± 1.1
C	7.0[b] ± 0.5	8.9[b] ± 2.8	16.4[a] ± 3.9
D	24.5[a] ± 2.0	19.9[a] ± 2.8	25.1[a] ± 3.9

[a,b] Means ± std dev (Steptoe, n = 10; Crystal, n = 5; Waxbar, n = 5) are reported on a dry weight basis. Steptoe was fractionated in a batch-type sieve. Crystal and Waxbar were fractionated in a commercial continuous-feed type sieve. Within rows, values followed by the same superscript letter are not significantly different ($P \leq 0.05$).

[c] Unfractionated barleys either dehulled, pearled or cleaned as described in text.

Batch-type sieving. Milled Steptoe barley and remilled coarse fractions (500 g/screen) were sieved for 1 hr in a custom-made set of 10 stainless steel screens (325-mesh, 30 cm × 60 cm) with trays for collecting fines. The stack of screens/trays was shaken on a rotary action shaker of a flour mill (C. W. Brabender Instruments Inc., South Hackensack, NJ). Fractions from each set of 10 screens and trays were combined and weighed. Coarse fractions were reprocessed.

Continuous-type sieving. Pin milled Crystal barley flour #5 (22.68 kg) and Waxbar barley flour (22.68 kg) were fractionated in a continuous feed sifter (Tru-balance sifter, model 511S, Great Western Mfg. Co., Inc., Leavenworth, KS) fitted with three screens (325-mesh, 60 × 60 cm) in series and fed at varying rates by a screw type feeder (model FDFM C/2, 2.54 cm outlet, Acrison Inc., Moonachie, NJ). Feed rate was determined 4 times during sieving by totaling the weights of two streams (fines and coarse) collected in 0.5-hr periods. Coarse fractions were reprocessed.

Analytical methods

Test sieving. As a measure of degree of grinding and effectiveness of separations, fractions (10.0g) were test sieved using 20 cm, U.S. Standard screens (50, 200 and 325 mesh) with openings of 300, 75 and 45 μm, respectively. The screens were shaken on a Ro-tap testing sieve shaker (W. S. Tyler Co., Cleveland, OH) for 1 hr. Nylon rings were used as sieving aids.

Composition. The various fractions were analyzed for ether extractives, ash and protein (N × 6.25) (AOAC, 1980). Total and soluble dietary fiber were determined by the methods of Prosky et al. (1984, 1988). Starch was determined using the AACC method 76-11 (1983) with minor modification by Knuckles et al. (1992b). Beta-glucans were determined by the methods of McCleary and Glennie-Holmes (1985) and McCleary and Nurthen (1986). The enzymes for β-glucans assay were supplied by Megazyme (North Rocks, Australia). The soluble and insoluble β-glucans were separated as described by Knuckles et al. (1992b) in a manner similar to that reported by Åman and Graham (1987).

Tocopherols and tocotrienols. The tocopherols (T) and tocotrienols (T3) collectively identified as tocols were analyzed by an HPLC method. Tocol extraction was by a method adapted from that of Peterson and

Qureshi (1993). The use of tetrahydrofuran/hexane as mobile phase was suggested by the work of Gapor et al. (1981). Sample (1.0g), ground to pass a 0.2 mm screen (Udy mill, Fort Collins, CO), was extracted in a sealed 12-mL tube with 10.0 mL methanol (magnetic stirrer for 30 min, 22–25°C). After centrifugation (2 min, 3000 × g), the extract (8.0 mL) was evaporated under vacuum in a 50-mL round bottom flask warmed in a water bath (30°C). The residue was extracted with 2.0 mL hexane (2.0 min) which was dried by shaking with 0.3g anhydrous Na_2SO_4 (2.0 min) and centrifuged (2.0 min, 3000 × g). Care was taken to reduce exposure to light and oxygen by using amber or red containers and sparging and blanketing solvents/extracts with argon.

The hexane extract (20.0 μL) was immediately injected on to a silica HPLC column (3.9 × 150 mm, 4 μm. Nova-Pak®, Waters Division, Millipore Corp., Milford, MA) and eluted using hexane/tetrahydrofuran (97.5/2.5) at 0.5 mL/min. Detector response for both tocols decreased as much as 10–20% in 2 hr. Tocols were quantitated by monitoring HPLC elution at 295 nm excitation and 330 nm emission and calculating concentrations using regression equations of peak area vs individual tocol concentration ($r^2 \geq 0.995$). The monitor was a luminescence spectrometer (model LS-5, Perkin Elmer, San Jose, CA) with output to a chromatographic data handling system (Hewlett Packard, Pleasanton, CA). Authentic α-, β-, δ-, and γ-tocol standards chromatographed alone and in mixtures with sample extracts were used for peak identification. Samples of authentic tocols were gifts from Drs. A. A. Qureshi and D. Peterson (USDA-ARS, Madison, WI). A reference sample of tocol-rich palm oil of known tocol concentrations was a gift from Dr. K. H. Loke (Palm Oil Research Institute, Kuala Lumpur, Malaysia). Data for the regression equations were generated from 20 μL injections (3) at four tocol levels. Aliquots (5, 10, 15, 20 μL) of a stock solution of mixed tocols in hexane was diluted to 1.5 mL with hexane. The stock solution contained, in μg/mL, α-tocopherol (571.2), α-tocotrienol (1267.6), β-tocopherol (150.0), γ-tocopherol (100.0), β-tocotrienol (180.3), γ-tocotrienol (646.0), δ-tocopherol (200.0) and δ-tocotrienol (172.6).

Statistical evaluations. Means, regression equations, and Tukey's pairwise means comparisons with critical range were calculated using statistical software version 3.2 (Systat Inc., Evansville, IN).

RESULTS & DISCUSSION

Milling

Hull removal from the Steptoe barley by the rice whitening machine was incomplete although weight (18%) removed as hull exceeded the theoretical weight (13.4%) removed by hand (Knuckles et al., 1992b). Complete removal of hull in the crease region of the kernel would remove substantial amounts of underlying endosperm. Others reported removing 20–30% of barley weight as hull fraction (Vose and Youngs, 1978; Foehse, 1991).

Heat, sufficient to melt a plastic (bag) collection bin liner, was generated during pin milling of dehulled Steptoe barley at an undetermined feed rate >16.2 kg/hr. By keeping the feed rate below 16.2 kg/hr, product in the collection bin did not exceed 40°C. Overheating was not apparent during pin milling of the coarse (>45 μm) Steptoe fractions at feed rates to 37.8 kg/hr or during hammer milling of Crystal and Waxbar barleys where feed rates to the PMCO hammer mill and Pulvacron were 164 and 350 kg/hr and 300 and 500 kg/hr, respectively.

Flours of comparable coarse particle (>45 μm, <300 μm) content were produced by the different mills (Table 1). Sixty to 85% of the particles in the flours were <150μm. The flours tended to have larger particles than those (66% <15 μm) produced by Vose and Youngs (1978) but smaller than those from roller milling (42% >250 μm) (Bhatty, 1993). Foehse (1991) recommended that barley be milled such that 90% of particles be <600 μm with >50% being >200 μm.

Sieving

Yields of β-glucan-enriched fraction D from the three barley cultivars obtained by batch- or continuous-sifting were not different (Table 2). These fraction D yields were in the range (17.5–24%) reported when hulled and hull-less barleys were processed (Knuckles et al., 1992b; Foehse, 1991). Yields of fractions A, B, and C from Waxbar were different ($P \leq 0.05$)

Table 3—Nitrogen, fat, ash and starch content (%, dry basis) of barley fractions[a]

Barley/Fraction	Nitrogen	Fat	Ash	Starch
Steptoe[b]				
Whole grain	1.99	2.95	2.54	68.90
Hull	1.41	3.40	6.74	27.99
Dehulled	2.02	2.57	1.82	78.44
A	1.97	2.17	1.55	90.69
B	2.04	2.67	1.66	84.24
C	2.07	3.43	2.00	78.27
D	2.05	3.71	3.73	55.10
Crystal[c]				
Pearled	1.78	2.90	1.33	79.54
A	1.54	2.58	1.13	86.75
B	2.68	2.67	1.36	85.24
C	1.50	2.71	1.38	81.58
D	2.38	4.17	2.53	62.54
Waxbar[c]				
Cleaned	1.55	2.51	2.23	80.46
A	1.51	1.92	1.63	88.57
B	1.57	1.90	1.90	88.32
C	1.66	2.41	2.21	66.33
D	2.09	3.40	3.14	47.03
LSD$_{0.05}$[d]	0.05	0.46	1.55	6.31

[a] Nitrogen, fat and ash, n = 2. Starch, n = 3. Moisture (all samples) = 9.3-10.8%.
[b] Pin milled then sieved in a batch-type sifter.
[c] Pin milled than sieved in a commercial continuous-feed sifter.
[d] Least significant difference.

Table 4—Dietary fiber and β-glucans content (%, dry basis) of barley fractions[a]

Barley/ Fraction	Dietary fiber			β-Glucans		
	Total	Soluble	Insoluble	Total	Soluble	Insoluble
Steptoe[b]						
Whole grain	22.32	3.64	18.68	4.36	2.04	2.29
Dehulled	17.18	6.00	11.18	5.76	2.91	2.83
A	4.24	1.03	2.97	0.80	0.41	0.36
B	9.19	5.48	3.25	3.20	1.52	1.36
C	15.03	6.11	8.62	6.53	3.30	3.14
D	45.08	19.88	22.20	18.32	9.51	9.51
Crystal[c]						
Pearled	13.72	3.51	6.62	5.73	1.66	3.70
A	5.06	1.28	2.46	1.46	0.42	0.59
B	6.51	2.15	3.72	5.62	0.82	1.53
C	11.74	3.63	6.39	5.26	1.74	3.24
D	43.10	9.90	16.38	17.55	7.60	9.48
Waxbar[c]						
Cleaned	13.93	4.73	8.23	5.98	1.77	3.58
A	5.29	2.75	4.60	1.99	1.21	1.46
B	6.48	3.12	5.90	2.21	1.05	1.62
C	15.46	7.58	11.00	5.88	2.34	4.58
D	36.90	17.27	21.28	15.75	6.41	9.41
LSD$_{0.05}$[d]	0.64	0.81	0.98	0.52	0.73	0.76

[a] Values were means of 3 triplicates
[b] Pin milled then sieved in a batch-type sifter.
[c] Pin milled then sieved in a commercial continuous-feed sifter.
[d] Least significant difference.

from yields of similar fractions from Steptoe and Crystal due to impeded passage of fine particles. Removal of the fine particles by test sieve (US Standard 325-mesh) resulted in calculated theoretical yields for Waxbar fractions A, B, and C (51.4, 17.7 and 5.8%, respectively) which were not different (P ≤ 0.05) from yields from Steptoe and Crystal barley.

During sieving, strong attractions between particles prevented movement of the fine (<45 μm) particles to and through screens. These particles tended to block screen openings and form either a caked mass or balls which rolled around on the screens. The use of felt covered pads and nylon rings on top of the screens and small plastic balls and nylon bristle brushes in the trays collecting the fines was essential for facile separation of fines. Ease of particle separation depended on barley variety and the amount of fines in the feed material.

Crystal was more easily fractionated in the continuous sifter than Waxbar. At a feed rate of ~3.5 kg/hr, coarse fractions from Crystal, with 54.5% fines before sieving, were almost fines free (<1%), whereas, the coarse fractions from Waxbar, with 51.3% fines before sieving, contained 45% fines. Difficulties in contin-

uous sieving were greatest when feed material had >25% of its particles as fines. When Waxbar, with 34% fines, was fed at 2.6 kg/hr, up to 75% of the fines remained in the coarse fraction. Reducing the feed rate to 1.1 kg/hr reduced the fines in the coarse fraction to 1–2%.

A more effective alternative than slowing the feed rate to attain separation, was to add back some previously separated coarse fraction (>45 μm particles). When Waxbar (39% fines) was diluted to 26% fines (2 parts feed to 1 coarse fraction) and sieved at 5.7 kg/hr, 25% of the material was collected as fines. Thus, feed rate of unfractionated Waxbar was increased 3.45 times while fines in the coarse fraction remained <1%.

The fine particle size (99% >45 μm and 85–90% <150 μm) of the β-glucan-enriched fraction D could affect the viscosity, water absorption and other factors contributing to the quality of food products to which it is added. For example, rice flour with fine particles had higher water retention and produced smaller denser loaves of bread than flour with larger particles (Nishita and Bean, 1982). Also, particle size of barley flour affected physicochemical and breadmaking quality (Bhatty, 1986; Posner, 1991). Particle size may have contributed to qualities of baked products containing a barley fraction similar to D (Hudson et al., 1992; Knuckles et al., 1992a).

Air classification

Milled Crystal barley flours #1–3 were fractionated into coarse and fine fractions using the small scale air classifier. Recoveries, as coarse fractions, were 28.9, 43.0 and 56.3%, respectively. The β-glucan contents (7.9, 5.8 and 6.2%, respectively) of these fractions were less than twofold higher than the 4.5% in the feed material. Two further millings and separations of Crystal flour #3 yielded 35% as coarse fraction D containing only 8.9% β-glucan. Separations made with the large air classifier using Crystal flour #4 contained less β-glucan than those with the small unit. Separation in the large air classifier, at 400 and 500 kg/hr, yielded coarse fractions (55.5 and 52.8%, respectively) containing 5.0 and 5.2% β-glucan. Test sieving showed that these coarse fractions contained 10–15% particles <45 μm in size with <2% β-glucan and fine fractions contained 5–15% particles >45 μm in size with 5–6% β-glucan. Thus, β-glucan enrichment did not occur. These limited studies suggest the possibility that fractions containing >10–12% β-glucan would be difficult, if not impossible, to produce by air classifiers although reports state that air classifiers can produce fractions with 16–24% β-glucan (Danielson et al., 1994; Foehse, 1991). One air classifier used by Foehse (1991) had screens in the air stream.

Composition

In most cases, protein, fat and ash were highest in fraction D and lowest in fraction A increasing in succeeding fractions (Table 3). Protein in fraction D was up to 40% higher than in corresponding dehulled barley but did not reach the two-fold increases obtained by air classification of grains (Vose and Youngs, 1978). Protein recovery in fraction D was similar for the three barleys (25–27%). Starch content was highest in the A fractions and decreased in succeeding fractions. Starch content (90%) in fraction A from Steptoe approached the 92% of high purity corn starch. Starch content of the fine fractions tended to be higher than in starchy fractions produced earlier during dry fractionation of grains (Knuckles et al., 1992b; Vose and Youngs, 1978; Pomeranz et al., 1971). Starch recovered in fraction D was similar for the 3 barleys (15–17%), but starch recovered in fraction A differed between Waxbar (25%) and Steptoe and Crystal (62%). Fraction B from Waxbar contained more starch (42%) than fraction B from Steptoe (18%) or Crystal (16%).

Total and soluble dietary fiber content of fraction D was 2.5 to 3.7 times that in dehulled barley (Table 4). The ratio of sol-

uble to insoluble fiber was similar for dehulled Steptoe, Crystal and Waxbar barleys (0.54, 0.53 and 0.57, respectively) but differed in the enriched fraction D from these barleys (0.90, 0.60 and 0.81, respectively). Dietary fiber levels in fraction D were higher than those (total, 20%, soluble, 6.9% and insoluble, 11.7%) in bran fractions produced by roller milling of several other barley cultivars (Bhatty, 1993) differing from those we used. Total dietary fiber recovered in fraction D was 63–66%. The other fractions contained 6–20% of dietary fiber depending upon barley and fraction.

Beta-glucans were also enriched by dry milling and sieving. Removal of hull from Steptoe barley increased β-glucan content from 4.4% to 5.2%. The hull contained only 0.5% β-glucan. Beta-glucan concentration was lowest in fine fraction A and increased in succeeding fine fractions B and C. The high recovery (61–78%) of total β-glucans in the coarse fraction D resulted in concentrations from 16–18% (Table 4). Forty to 50% of the β-glucan was soluble in water. The β-glucan concentrations in D fractions were higher than the 5–12.5% in barley bran and shorts from roller milling (Bhatty, 1992; 1993). The ratio of soluble to insoluble β-glucans differed among dehulled Steptoe (1.03), Crystal (0.45) and Waxbar (0.49). This ratio increased with processing of Crystal and Waxbar but not with Steptoe. Selection of barley based on attainable soluble β-glucans content has been suggested by reports that soluble β-glucans may be a better predictor for lowering serum cholesterol than dietary fiber or total β-glucans (Newman et al., 1992).

Fraction D could be used to increase fiber and β-glucans content or possibly replace fat in food products. When a similar fraction was substituted for part of the flour in breads, muffins and pasta, panelists rated the products acceptable (Hudson et al., 1992; Knuckles et al., 1992a). An oat product containing β-glucan-amylodextrins reportedly could reduce fat content of desserts (Inglett, 1990). Consumption of such fortified products should reduce the risk of coronary heart disease and aid in control of diabetes. Published studies indicate β-glucans lower serum cholesterol and dietary fiber lowers blood glucose response in diabetics. Such health benefits may be related to factors such as viscosity which affects the gastrointestinal system (Newman et al., 1989b). Consideration of barley cultivar to be processed may be prudent since viscosities of β-glucans differ among cultivars (Xue et al., 1990; Bhatty, 1992).

Tocopherol and tocotrienol concentrations differed among barley fractions (Table 5). Generally, tocol concentrations in Steptoe fractions were higher than those in similar fractions of Crystal or Waxbar. Peterson and Qureshi (1993) reported genotype differences for most tocols. Generally, the highest tocopherol levels were found in the Steptoe hull fraction possibly because the germ and aleuron were removed with the hull fraction. Individual tocotrienol levels were higher than corresponding tocopherol levels. These trends in tocol concentration were in agreement with results reported by Peterson (1994). Although tocols were concentrated in various fractions, they were not concentrated to levels (205 μg/g) attained during pearling of waxy hull-less barleys but were similar to concentrations found in roller milling fractions (Wang et al., 1993). Tocol concentrations were moderated since recoveries in any one fraction were <50%. The highest total tocols recoveries were in fraction A from Steptoe (44%) and Crystal (50%). About 1/3 of the tocols were recovered in fraction D. Total T3 concentrations in fraction D from Steptoe, Crystal and Waxbar were 73.4, 41.1, and 64.5 μg/g, respectively. The highest levels approached the 80–100 μg/g tocotrienols in brewer's grain fractions which reduced β-hydroxy-β-methylglutaryl Coenzyme A reductase activity and reduced levels of total serum cholesterol and low density lipoprotein (Qureshi et al., 1991). The tocols may contribute to reduced lipid oxidation which alters sensory properties/nutritive values of fats. Tocotrienols (200 μg/g) had significant antioxidative properties (Gapor et al. 1989) and tocopherols have reduced lipid oxidation (Burton and Traber, 1990).

CONCLUSIONS

BARLEY CAN BE MILLED by hammer or pin mills and sieved to produce fractions enriched in dietary fiber, β-glucans and tocols. Separation of fine particles requires recycling of coarse fraction to the feed, or use of physical sieving aids (felt pads, brushes). Under our conditions, air classification was a less effective particle separation technique than sieving.

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Color Retention in Red Chile Powder as Related to Delayed Harvest

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ABSTRACT

Color retention in chile powder was studied using two mildly pungent long red chile (*Capsicum annuum* L.) cultivars, 'New Mexico 6-4' and 'NuMex R Naky'. Pods of different maturities were harvested immediately after the first severe fall freeze and again 4 and 8 wk after the freeze. After each harvest pods were dried, ground, and immediately analyzed for color. Powder was stored at 2°C and re-analyzed for color at 4 wk intervals. Color loss in whole pods from the field and the stored powder showed linear and quadratic trends. Initially, delaying harvest resulted in color loss in red chile but color in powder from later harvests was more stable and was retained better after 8 wk storage than powder from the first harvest after the same length of time.

Key Words: red chile, delayed harvest, red color, pod maturities

INTRODUCTION

CHILE PEPPER (*Capsicum annuum* L.) is an important food and flavor ingredient in many countries throughout the world. New Mexico ranks first in U.S. production of this vegetable. In the United States there have been increased markets for ethnic foods such as traditional southwestern dishes which contain chile. Red chile also adds color to foods which comes from the carotenoid content of the peppers. Understanding factors affecting color in peppers is of interest to spice and food coloring industries worldwide.

Pod maturity studies consistently have shown more mature pods to have higher total and more varied forms of carotenoids (Rahman and Buckle, 1980). Simpson et al. (1974) found phytoene, phytofluene, α-carotene, and lutein are not in fully mature pepper pods; while less mature peppers contained these carotenoids but lacked other carotenoids. Several studies have shown cultivar differences in both types and total carotenoid contents of peppers (Davies et al., 1970; Almela et al., 1991).

The loss of color in chile powders has been attributed to autoxidation of carotenoids (Carnevale et al., 1980). Such degradation is affected by the level of natural antioxidents, ascorbic acid and tocopherol (Lantz, 1943; Rahman et al., 1978; Biacs et al., 1992), the relative stabilities of different types of carotenoids, lipid and moisture content (Ramakrishnan and Francis, 1979a, b, 1980).

Limited processing facilities and adverse weather conditions may prolong harvesting seasons. In New Mexico harvesting normally begins in late September but may extend into February. Few studies have compared loss of color in whole pods left in the field after the first killing frost with that of powder from pods harvested immediately after the first killing frost. Isidoro et al. (1990) reported that color loss in whole pods left outside was less than in powder stored in the dark at 4°C. No published studies have reported color loss in powder from pods left in the field for extended periods after the first killing frost. Using pods

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grown during the 1983 growing season in the Mesilla Valley, our objective was to determine any effects of pod maturity, cultivar, time of harvest and length of storage on the stability of color in red chile powder.

MATERIALS & METHODS

'NUMEX R NAKY' AND 'NEW MEXICO 6-4' were planted in the Mesilla Valley of southern New Mexico on March 14, 1983 and managed under a growing regime approximating practices used by commercial growers. The two cultivars were selected because they are releases of New Mexico State University developed for the commercial market. 'Nu Mex R Naky' was specifically developed to have a high carotenoid content for production of red chile (Nakayama and Matta, 1985). On September 12, 1983 six uniform plots (replicates) of each cultivar were designated and 75 pods of three maturity stages (25 for each maturity) in each plot were tagged for later harvest. The pod maturities were (1) early pod set (pods produced on the first three branching nodes and bright red at tagging time); (2) mid-season pod set (pods on fourth through sixth branching nodes and chocolate and green color); and (3) late pod set (mature green fruit developing along the upper dominant branches). Harvesting of tagged pods began after the first killing frost on November 9. Five pods from each plot were harvested at 28 day intervals and oven dried at 60°C. After 5 days at 60°C, pods were washed in a detergent solution to clean off soil and other debris that might interfere with carotenoid extraction or detection, rinsed and redried for 2 more days.

Dry pods with stems attached were ground in a Wiley mill to pass a 1 mm screen. A representative powder sample of each plot was placed in a small lab dryer (60°C) for ~16 hr prior to color analysis. The remainder was stored in brown plastic vials (2.8 × 7 cm) at 2°C for future color analysis at 28-day intervals.

Dried chile powder from each sample was analyzed for red color by a modified procedure of the American Spice Trade Association's (ASTA) official analytical method (Horwitz, 1980). Dry powder (~70 mg) was placed in 125 mL flasks and extracted by soaking in 50 mL of

Color value (ASTA units) of chille powder immediately after harvest

Cultivar	Maturity		MEAN
1	1		94.83 c
	2		138.33 b
	3		135.83 b
2	1		120.67 b
	2		171.67 a
	3		178.50 a

Color value in ASTA units

Legend: Cultivar : 1: New Mexico 6-4 2: Numex R Naky

Pod Maturity: 1: Early pod set 2: Mid-season pod set 3: Late pod set

Fig. 1—Cultivar and time of pod maturity effects on color value in red chile powder immediately after harvest. Means followed by the same letters are not statistically significant based on Waller-Duncan's T test (K-ratio = 100).
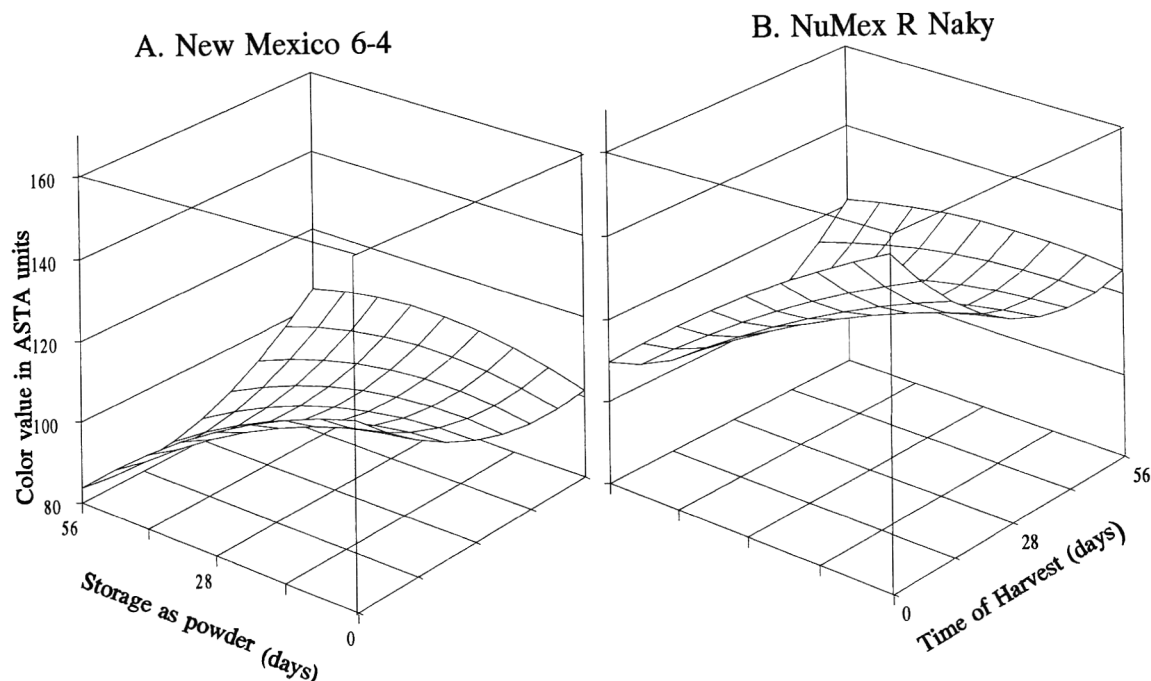
A. New Mexico 6-4

B. NuMex R Naky

Fig. 2—Cultivar effects on the two factor [Days of storage (S) × Harvest time (H)] quadratic response surface plots on red chile powder color. Response surface model for cultivar 'New Mexico 6-4' (A) Color value = 124.7 − 1.02 H − 0.34 S + 0.011 H^2 − 0.007 S^2 + 0.014 HS (R^2 = 0.95); 'NuMex R Naky' (B) Color value = 158.1 − 1.53 H − 0.64 S + 0.017 H^2 − 0.004 S^2 + 0.014 HS (R^2 = 0.96).

acetone for 4 hr. A sample of the acetone containing carotenoids was placed in a cuvette and absorption recorded at 460 nm with a spectrophotometer. The ASTA values were determined by comparing the absorbance values of each sample with the absorbance of 0.4966 of a National Bureau of Standards (NBS) filter. The comparison was made by the equation: ASTA = absorbance × NBS × 4.1 × sample wt.

The data were analyzed as a split-plot design. Two cultivars were designated as the mainplot in a randomized complete block design with six replications and the three stages of maturity were treated as the subplots. Time of harvest and length of storage were treated as two repeated measures. The statistical significance of each factor and their interactions were tested by multivariate test (Wilk's Lambda) and repeated measures ANOVA F-test with H-F adjustment (Littell et al., 1991).

RESULTS & DISCUSSION

EACH POD MATURITY of 'NuMex R Naky' had more color than the corresponding pod maturity in 'New Mexico 6-4' (Fig. 1). In this study 'NuMex R Naky' had higher ASTA values than 'New Mexico 6-4'; however, this was only true of the early and mid-season pod sets. If more late season pods than earlier set pods were mixed in the harvest of 'NuMex R Naky' than for a comparable harvest of 'New Mexico 6-4', then ASTA values for 'New Mexico 6-4' might equal or slightly exceed 'NuMex R Naky'. Early and mid season pod sets were not different from each other in either cultivars. Thus mid-season pods had enough time to fully ripen. Both mid-season and late season pod sets of 'NuMex R Naky' had higher ASTA values than any of the pod sets of 'New Mexico 6-4'. This suggested that 'NuMex R Naky' had a greater potential for cartenoid synthesis than 'New Mexico 6-4', which was confirmatory of Nakayama and Matta (1985). Both early and mid-season pod sets were leathery and wrinkled just before the first killing frost. The late pod set was still succulent and it is likely that carotenoid synthesis was not complete in this set pod.

Both time of harvest and length of storage had significant effects on color intensity in chile powder. Regression analysis exhibited significant quadratic patterns of color loss between analyses for either time of harvest or length of storage (Fig. 2 and 3). During the first month, all pod maturities and both cultivars lost color more rapidly in whole pods in the field than in powder in the dark at 4°C (Fig. 2 and 3). After 1 mo, color loss in powder from the first harvest accelerated while color in the

whole pods outdoors appeared to stabilize. The only exception to the color stability in stored powder from the second and third harvests occurred when the late pod set powder lost color during 2 mo storage. However, the color loss occurred at a reduced rate compared to powder from the first harvest (Fig. 3a).

The color loss of chile powder from the first harvest was expected in confirmation of findings by other researchers (Lease and Lease 1956; Carnevale et al. 1980). The color stabilization in whole field pods or powder from 1 or 2 mo delayed harvests was unexpected. Research has been done on stabilizing color in pepper powders by adding synthetic or natural antioxidants that occur in peppers. These antioxidants are ascorbic acid and α-tocopherol. But, according to studies by Lantz (1943) and Biacs et al. (1992), ascorbic acid starts to degrade rapidly at the same time that carotenoid synthesis starts to increase rapidly. Alpha-tocopherol rapidly degrades at about the same rate and time as carotenoids (Biacs et al., 1992). Specific composition of carotenoids is probably responsible for greater color stability in powder from the more mature pods. Rahman and Buckle (1980) found more mature peppers had a higher content and greater variety of cartenoids. Philip et al. (1971) found more stable red coloration in paprika with higher capsanthin concentration. However, carotenoid composition could not be responsible for the greater stability in later harvest because carotenoid synthesis probably ceased before first harvest. Some enzymatic or nonenzymatic reactions probably occurred in the field that altered some carotenoids in the early and mid-season pod sets but not in the late pod set. Such reactions may have been prevented from occurring in powder from pods in the first harvest either by disruption of tissue caused by grinding or by denaturation of enzymes responsible for this unknown reaction. The enzymes could conceivably have been denatured by the 60°C in the dryer.

CONCLUSION

NEW MANAGEMENT PRACTICES need to be considered for maximum color and its stability. To achieve maximum color stability, harvest should be delayed between 1 and 2 mo after frost. Growers should harvest only the pods that are wrinkled and leathery and delay harvest for 1 to 2 mo after metabolism in the plants has ceased. Planting cultivars known to have a high con-

A. Early Pod set   B. Mid-Season Pod set   C. Late Pod set
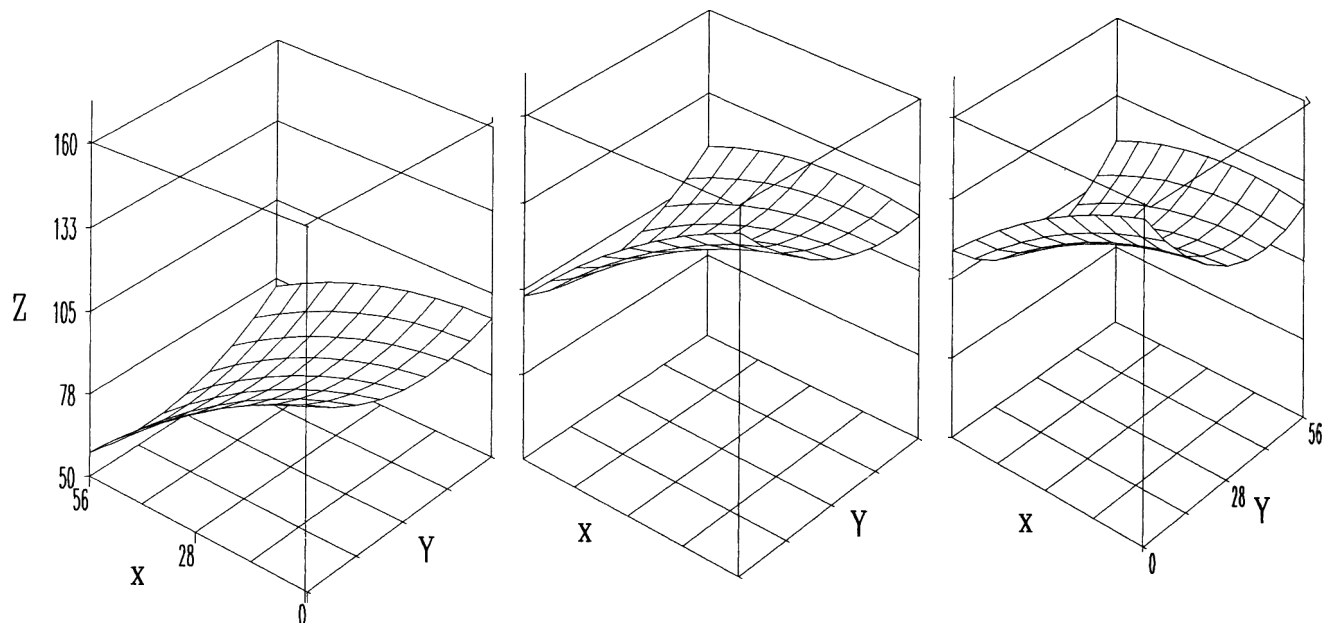
Z - Axis : Color value of powdered chilli pods in ASTA units
X - Axis : Days of Storage as powder
Y - Axis : Time of Harvest in days

Fig. 3—Effects of time of pod set on two factor [Days of storage (S) × Harvest time (H)] quadratic response surface plots on red chile powder color. Response surface models for Early pod set (A) Color value = $108.3 - 0.88\,H - 0.50\,S + 0.014\,H^2 - 0.007\,S^2 + 0.009\,HS$ ($R^2 = 0.89$); Mid-season Pod set (B) Color value = $156 - 1.36\,H - 0.53\,S + 0.014\,H^2 - 0.007\,S^2 + 0.015\,HS$ ($R^2 = 0.97$); Late pod set (C) Color value = $157.2 - 1.87\,H - 0.36\,S + 0.023\,H^2 - 0.007\,S^2 + 0.011\,HS$ ($R^2 = 0.99$).

centration of carotenoids in the ripe fruit should also be encouraged. Cultural practices which encourage late maturing fruit, with color characteristics analogous to late-set pods described here should be discouraged. Some practices that contribute to late set include: late planting, excessive and/or late season applications of nitrogen fertilizer, and harvesting a green crop before allowing subsequent fruit to mature red.

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Model for Gas Exchange Dynamics in Modified-Atmosphere Packages of Fruits and Vegetables

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ABSTRACT

A model for atmosphere in a package containing fresh fruits was analyzed theoretically and validated by experiments with red bell pepper fruit. The model was based on two processes: fruit respiration and film permeability. Mathematical analysis showed that when rates of O_2 consumption and CO_2 evolution due to respiration are equal, and film permeability to CO_2 is greater than that to O_2, the time course curve of CO_2 concentration has a maximum. In a closed system, the time course of the sum of the gases could indicate a change of respiratory quotient. These results are independent of model for respiration. To predict extent of overshoot and for a computerized simulation, the equation of Michaelis-Menten type with noncompetitive inhibition was adopted to describe respiration. Utilizing computer simulations enabled evaluation of film specifications and package dimensions best for a given commodity.

Key Words: dynamic model, modified atmosphere, respiration, enzyme kinetics, bell pepper

INTRODUCTION

THE KEEPING QUALITY of fruits and vegetables is affected by temperature, and by concentrations of oxygen, carbon dioxide and water vapor in surrounding atmospheres (see reviews by Tomkins, 1962; Kader et al., 1989; Gorris and Peppelenbos, 1992). Modified-atmosphere (MA) packaging is used to prolong storage life of perishable commodities (Ben-Yehoshua et al., 1985; Kader et al., 1989). The search for optimal parameters for an MA system may be assisted by a theoretical analysis which can predict temporal changes in package atmosphere in terms of package characteristics and temperature.

Several models of MA packaging systems have been discussed (Jurin and Karel, 1963; Hayakawa et al., 1975; Cameron et al., 1989; Mannapperuma et al., 1989; Emond et al., 1991). In most cases they were restricted by choice of empirical equations fitted to observed changes in gas concentration. The models of this type are not based on a consideration of biophysical mechanisms for the processes in MA packages and have utilized different equations in different studies. This makes difficult any comparison and application of results.

Gas diffusion and fruit respiration are the 2 main processes which affect the package atmosphere. Fick's diffusion law was found to govern gas exchange in apples and several other fruits (Burg and Burg, 1965). The respiration of fruits and vegetables was treated in terms of enzyme kinetics by Burton (1978) and Tucker and Laties (1985). Chevillotte (1973) discussed the interaction of respiration and diffusion in oxygen uptake by different biological materials. One of these processes may predominate in different cases. Several attempts have been made to apply principles of enzyme kinetics to MA package modeling (Lee et al., 1991; Ben-Yehoshua et al., 1994; Cameron et al., 1994; Joles et al., 1994; Talasila et al., 1994). The action of CO_2 as a suppressor of respiration was treated by Lee et al. (1991) as noncompetitive inhibition in the enzymatic Michaelis-Menten reaction. They performed a numerical solution of the mathe-

matical equations for simulation of gas composition in the MA packaging system. The values of Michaelis-Menten parameters needed for the numerical calculations are usually estimated by linear plot methods. In many cases such methods provide ineffective estimates of parameters, as was shown by Reich (1970), Cornish-Bowden (1976) and Ross (1981). More comprehensive analysis of the mathematical model could improve the accuracy of prediction.

Most of the previous works on MA packaging concentrated on analysis of in-package atmospheres under steady state (Mannapperuma et al., 1989; Cameron et al., 1994; Joles et al., 1994; Talasila et al., 1994). However, in many cases it takes a long time to reach gas equilibrium. During the transient period, until steady state is established, the produce in a package is exposed to an atmosphere different from steady-state. In certain cases, the transient period might be as prolonged as the entire storage time, while steady state is reached only by the end of storage (see, for example, Geeson et al., 1985) or would not be reached at all. The analysis of the transient period is important for such systems in order to predict the level of O_2 and CO_2 in the course of time, as well as to estimate the time necessary to approach steady state.

Our objective was to perform a theoretical analysis of gas exchange dynamics in an MA package followed by computer simulation of the system. This analysis allowed us to study in a general way the time course of oxygen and carbon dioxide concentrations and to determine specific characteristics of system kinetics. Nonlinear methods were applied to estimate the Michaelis-Menten parameters from observations under conditions of a closed system. To validate the model, experimental imitation of the MA package was undertaken. Red bell pepper was used as an example of a non-climacteric commodity. The absence of climacteric rise in the respiratory process simplified the system for establishing the proper model for gas exchange dynamics. Although packaging in plastic film extends storage life of bell peppers primarily by alleviation of water stress (Ben-Yehoshua et al., 1983), combining the optimal relative humidity with desirable concentrations of O_2 and CO_2 would provide additional benefits (Polderdijk et al., 1993, Rodov et al., 1995).

MATERIALS & METHODS

MATURE RED PEPPERS of the Maor cultivar were taken directly from the field or from the packing house. Fruit did not receive any postharvest treatment except washing.

Closed system

A closed system was used for estimation of oxygen uptake and carbon dioxide evolution. Fruit of known weight and volume were placed in 1-L glass jars, one fruit/jar. The jars were plugged tightly with massive rubber stoppers and submerged under water in a thermostatic bath at 20°C in order to prevent gas leakage.

To check the carbon dioxide effect on respiratory activity of the fruit, calcium hydroxide was added to some of the jars as CO_2 absorber. Sealed bags of gas-permeable spun-bonded polyethylene (Tyvek, Du Pont Co., Geneva, Switzerland) containing 10g of $Ca(OH)_2$ were placed on the bottom of those jars. Direct contact of the fruit with the CO_2 absorber was prevented by a perforated plastic Petri dish placed over the Tyvek bag.

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Three tubes were installed through the jar stopper. The central one was 4–5 cm above the stopper surface, tightly plugged at the upper end with a rubber septum 1 cm thick. Gas samples of 5 mL volume were withdrawn through this septum using gastight 20 mL glass syringe with a 0.5 × 16 mm needle. The two other tubes were connected through a Luer joint with two 35 mL plastic syringes each mounted vertically on top of the stopper. To ensure gas-tightness of these syringes, their plungers were covered with water. The syringes were used to prevent formation of negative pressure in the system due to regular sampling in order to ensure the accurate pressure and prevent leakage during syringe-sampling. After each sampling, the plunger in one syringe was lowered by 5 mL, compensating for the reduction in amount of gas in the system, and fixed with the splint. The homogeneity of the atmosphere within the whole system (jar and two syringes) was maintained by pumping air with syringe plungers. The partial vacuum formed in the jars with $Ca(OH)_2$ as a result of CO_2 absorption, was compensated for by injecting nitrogen to reach pressure equilibrium before each sampling.

Oxygen and carbon dioxide concentrations in the gas samples from the closed system were determined using a Packard 7500 gas chromatograph with thermal conductivity detector equipped with a Porapac column, using helium as carrier gas.

Plastic package imitation

In order to check experimentally the theoretical predictions of atmosphere changes, the modified-atmosphere package was simulated. A dome-shaped chamber, 1.6 L, with the open side 20 cm in diameter, made of gas-impermeable 0.4-cm-thick rigid plastic with flat 1.5-cm margins, was used to simulate an MA package. A low-density polyethylene (LDPE) film, 40 µ thick, was stretched over the open side of this chamber between two rubber rings of 1.5 cm width and covered with another rigid plastic ring of the same dimensions. The rings were lubricated with silicone gel to ensure gas-tightness and the system was fastened with double clips. This installation was tested for leakage by substitution of plastic film with gas-impermeable material and injecting the gas mixture of known concentrations. These concentrations were maintained at the same level for one week. There were three openings to the chamber: at the bottom it was connected by a rubber tube to a water manometer in order to monitor the pressure changes in the chamber during the experiment. The two other openings were inlet and outlet of air connected by rubber tubes to the gas analyzer through a small external peristaltic pump, DYMAX MK1 (Charles Austen Pumps Ltd., Byfleet, Surrey, UK). This system was in continuous operation without interruption during each experiment.

Gas analysis in the package imitation was accomplished using a Servomex 1450B instrument (Servomex, Crownborough, Sussex, UK). Oxygen concentration was determined in a magneto-dynamic cell of the gas analyzer by measuring the paramagnetic susceptibility of the sample gas. Carbon dioxide was measured by infrared absorption using a single beam dual wavelength technique. Gas concentrations were recorded digitally. The gas analyzer was calibrated prior to each experiment using known concentrations of O_2 and CO_2.

Two freshly picked mature red peppers of known weight and volume were placed in the chamber, closed as described above and kept for 2 wk. The first measurement was made 2–3 hr after closing the chamber; subsequently, gas levels were measured two or three times a day. Between measurements, the air movement between chamber and gas analyzer was interrupted by clamps. Whenever gas levels were recorded, the clamps were removed and the pump was turned on for a few minutes until the Servomex readings were stabilized. Then the pump was turned off and after 3–4 min the O_2 and CO_2 concentrations were recorded. The experiments were conducted at 20°C and 85% relative humidity (RH). The experiments were repeated four times during 1992/93 and 1993/94 seasons. Film permeability to oxygen and carbon dioxide was determined by the concentration-increase method (Taylor et al., 1960).

Numerical calculations

The numerical calculations were performed on a Macintosh computer using JMP (SAS Institute Inc., 1989) software for the parameter estimations and STELLA (High Performance Systems, Inc., 1992) software for dynamic simulations. Base units of the International System of Units, SI, (see Downs, 1988) were used throughout the study, excluding time. For graphical representation, as well as for simulations, it was much more convenient to give time in hr.

SYSTEM ANALYSIS & MODEL DESCRIPTION

THE CONCENTRATIONS of oxygen (C_1) and carbon dioxide (C_2) in the atmosphere of the package containing fruit are determined by two main processes: fruit respiration and permeation of gases through the plastic film. The package permeability to gases was approximated by Fick's law. The respiration rate is known to depend on both C_1 and C_2 (Kader et al., 1989). Generally, two different respiration rates/unit of commodity weight should be considered: for O_2 consumption, $r_1(C_1, C_2)$, and CO_2 evolution, $r_2(C_1, C_2)$. The gas exchange kinetics are described by the following equations:

$$dC_1/dt = [-r_1(C_1,C_2) \, W + p_1A \, (C_{1A} - C_1)/\Delta X]/V \quad (1)$$

$$dC_2/dt = [r_2 \, (C_1,C_2) \, W - p_2A \, (C_2 - C_{2A})/\Delta X]/V \quad (2)$$

where W is the commodity weight; V is the free volume of the package atmosphere; A is the surface area of the package; ΔX is the film thickness; p_1 and p_2 are film permeability coefficients to O_2 and CO_2, respectively; and C_{1A} and C_{2A} are respective concentrations in the external atmosphere.

To represent the respiration rate as a function of concentration in terms of enzymatic reaction, a Michaelis-Menten-type equation was used, with O_2 in the place of substrate and the product CO_2 acting as noncompetitive inhibitor.

$$r(C_1,C_2) = v_m \, C_1/(K_M + C_1 + K_iC_1C_2) \quad (3)$$

where $r(C_1, C_2)$ is the respiration rate; v_m is the maximal rate of Michaelis-Menten reaction, and K_M is the Michaelis constant defined as substrate concentration providing the reaction rate of $v_m/2$; and K_i is the equilibrium constant of the enzyme-substrate-inhibitor complex formation (defined as the reciprocal to the constant used by Lee et al., 1991). The parameters v_m, K_M and K_i are temperature-dependent (Cornish-Bowden, 1976; Volkenstein, 1977).

RESULTS & DISCUSSION

EQUATIONS (1) and (2) are nonlinear differential equations for two interdependent variables, $C_1(t)$ and $C_2(t)$. This system cannot be integrated analytically in its general form. To perform numerical calculations, the functions $r_1(C_1, C_2)$ and $r_2(C_1, C_2)$ must be specified and the numerical values of the parameters involved in the equations must be known. However, some general features of the solution, revealing a relationship between variables $C_1(t)$ and $C_2(t)$, can be studied prior to this specification.

As a first approximation we treated the interval of concentrations where the respiratory quotient (RQ) could be approximated as one, i.e., the case $r_1(C_1, C_2) = r_2(C_1, C_2)$. Summation of Eq. (1) and (2) in this case leads to

$$dC_2/dt + dC_1/dt = P_1C_{1A} + P_2C_{2A} - P_1C_1 - P_2C_2 \quad (4)$$

where P_1 and P_2 are characteristics of the MA system permeability defined as following combinations of permeability coefficients of film and package parameters

$$P_{1,2} = p_{1,2}A/V\Delta X \quad (5)$$

Equation (4) does not contain a respiratory term and, as a linear combination of Eqs. (1) and (2), it may replace one of them in the system describing the gas kinetics in MA packaging, subject to RQ = 1. A study of the behavior of the system with time (transient period) may be important in the case when steady-state methods are not applicable.

Transient period

Overshooting effect. Equation (4) is a linear differential equation for the variable $C_2(t)$. Considering $C_1(t)$ and its derivative as implicit functions of t, integration of Eq. (4), subject to the initial conditions $C_1(0) = C_{1A}$ and $C_2(0) = C_{2A}$, leads to the
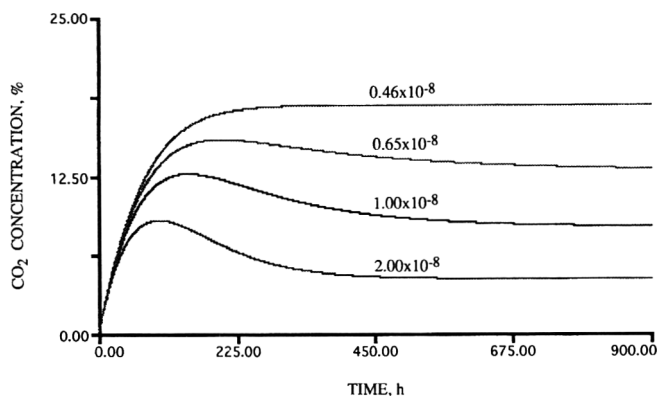
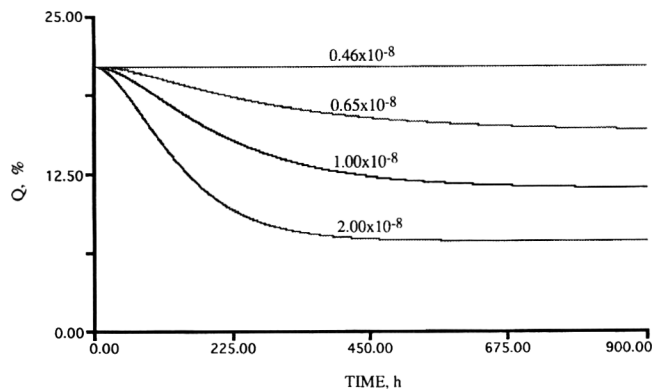**Fig. 1**—**Time course of carbon dioxide concentration predicted by model calculations for different values of coefficient of film permeability to CO_2, p_2 = (2.0×10^{-8}, 1.0×10^{-8}, 0.65×10^{-8} and 0.46×10^{-8}) m^2/hr, when the permeability to O_2, p_1 = 0.46 $\times 10^{-8}$ m^2/hr.**

Fig. 3—**Time course of the sum of oxygen and carbon dioxide concentrations, Q, predicted by model calculations for different values of coefficient of film permeability to CO_2, p_2 = (2.0×10^{-8}, 1.0×10^{-8}, 0.65×10^{-8} and 0.46×10^{-8}) m^2/hr, when the permeability to O_2, p_1 = 0.46 $\times 10^{-8}$ m^2/hr.**

Fig. 2—**Time course of oxygen and carbon dioxide concentrations and their sum observed in a closed system.**

following relationship between CO_2 and O_2 concentrations

$$C_2(t) = C_{2A} + (P_1/P_2)(C_{1A} - C_1(t)) - \qquad (6)$$
$$(P_2 - P_1)(1/P_2) \exp (-P_2 t) \int_0^t \exp (P_2 t) dC_1(t)$$

The following analysis of Eq. (6) shows that $C_2(t)$ has a maximum. The function has the maximum at the instant of time, where its derivative $dC_2(t)/dt$ changes its sign. Differentiation of Eq. (6) yields

$$dC_2/dt = - dC_1/dt + (P_2 - P_1) \exp \qquad (7)$$
$$(-P_2 t) \int_0^t \exp (P_2 t) dC_1(t)$$

If the oxygen concentration diminishes with time, then dC_1/dt is negative and the first term in the right hand side of Eq. (7) is positive. The second one is also positive, if $P_2 < P_1$. In this case $dC_2(t)/dt$ does not change its sign, and $C_2(t)$ has no maximum. As can be seen from Eq. (7), the condition of a maximum existence is: $P_2 > P_1$, i.e., the permeability to CO_2 has to be greater than that to O_2. This is the situation with practically all films used for MA packaging. The greater the difference $P_2 - P_1$, the more distinguishable the maximum. Figure 1 demonstrates calculated CO_2 kinetics in MA packaging with four hypothetical films having the same oxygen permeability coefficient (p_1 = 0.46 $\times 10^{-8}$ m^2/hr) and different CO_2-permeability coefficients (p_2 = 2.0×10^{-8}, 1.0×10^{-8}, 0.65×10^{-8}, and 0.46 $\times 10^{-8}$ m^2/hr). The maximum is absent on the last curve, where CO_2-permeability is equal to O_2-permeability.

Sum of O_2 and CO_2 concentrations as an important characteristic of the system

If $P_1 \geq P_2$, then the sign of the derivative dC_2/dt is positive and does not change throughout the time, i.e., $C_2(t)$ has no maximum. This is the situation, for instance, with a closed system where $P_1 = P_2 = 0$. In this case, the sum of the concentrations $Q = C_1(t) + C_2(t)$, is a constant. This statement follows also directly from the Eq. (1) and (2) when $P_2 = P_1 = 0$ and $r_1(C_1, C_2) = r_2(C_1, C_2)$.

The behavior of the sum Q may be utilized for the very important objective of rapid and convenient indication of change in RQ, without calculation of respiration rates. The condition $r_1(C_1, C_2) = r_2(C_1, C_2)$ is equivalent to RQ = 1. In a closed system, Q does not change when this condition is fulfilled. Rising Q in a closed system means that $r_2(C_1, C_2)$ becomes greater than $r_1(C_1, C_2)$, pointing to initiation of the anaerobic processes. Experimental data changes with the course of time for oxygen, carbon dioxide and their sum observed in the closed system containing red bell peppers were followed (Fig. 2). The level of Q remained constant for ≈ 150 hr, after which it rose sharply. It may be concluded that the condition $r_1(C_1, C_2) = r_2(C_1, C_2)$ was no longer fulfilled, pointing to the change of RQ. In the plastic film packages, the sharp breakage of the time course of Q also indicates the change of RQ and the switch to anaerobiosis. Such a rise indicates accelerated fermentation which is usually associated with anaerobiosis. These conditions are important because of risk of the development of anaerobic pathogenic microbial populations such as *Clostridium botulinum*.

As follows from Eq. (7), if $P_2 > P_1$ (as is the case of the package using non-perforated films), $Q(t)$ diminishes with time. A sigmoidal form is predicted for this curve because $dQ/dt = 0$ at time $t = 0$, since the initial gas gradients are equal to zero. The larger the difference $P_2 - P_1$, the stronger the deviation of $Q(t)$ from the horizontal line. Figure 3 demonstrates the set of curves $Q(t)$ for different values of p_2 ($p_2 = 2.0 \times 10^{-8}$, 1.0×10^{-8}, 0.65×10^{-8}, and 0.46 $\times 10^{-8}$ m^2/hr), whereas O_2-permeability coefficient was $p_1 = 0.46 \times 10^{-8}$ m^2/hr. Approach of the curve $Q(t)$ to a horizontal line may point to leakage in the system. The decline of $Q(t)$ from its initial level should result in gradual development of a pressure deficit and/or reduction of package volume. These changes were observed during our experiments. The pressure deficit reached <2% of the atmospheric pressure after 400 hr of observations. As a first approximation, the effect of these changes on model accuracy was assumed to be negligible.

Steady-state concentrations

The steady-state condition corresponding to $dC_1/dt = dC_2/dt = 0$, is a limiting case of the kinetic equations analyzed above.
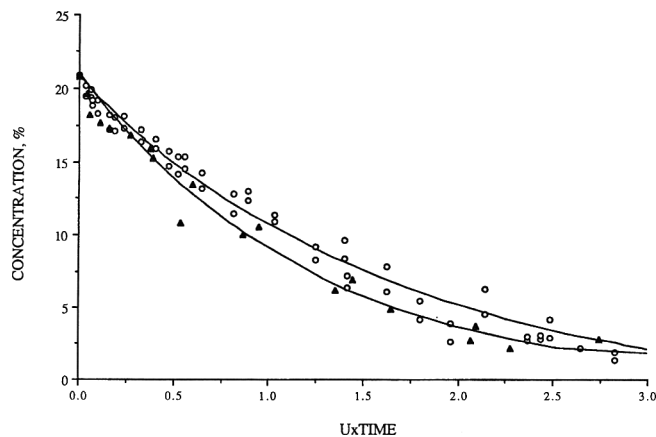
Fig. 4—Observed evolution of oxygen concentrations in a closed system with (triangles) or without (circles) Ca(OH)₂ as carbon dioxide absorbent. The curves were obtained from the model predictions by nonlinear fitting to experimental points: the lower curve was obtained from solution of the Michaelis-Menten equation without the inhibition term; the upper curve corresponds to the case where the inhibition was considered. Time multiplied by the ratio U = 100W/V was used instead of t to bring together the experimental data obtained with the packages containing fruit with different weights and volumes.

Under steady-state conditions, Eq. (4) gives a linear relationship between steady-state concentrations of O_2 and CO_2, denoted as C_1 and C_2, respectively:

$$C_2 = C_{2A} + (C_{1A} - C_1)\, p_1/p_2 \qquad (8)$$

This equation and its applications were analyzed in details by Mannapperuma et al. (1989), who showed, that important information concerning the package system can be obtained from this equation alone, without specifying respiratory equations.

For $dC_1/dt = 0$, a combination of Eq. (1) and (3) gives

$$100W\ \Delta X\ v_m C_1/(K_M + C_1 + K_iC_1C_2) = p_1A\ (C_{1A} - C_1) \qquad (9)$$

The factor 100 is to be used to fit the dimensions (see Nomenclature). Now, the set of Eq. (8) and (9) allows one to calculate the steady-state concentrations, C_1 and C_2. For the case where no inhibition occurs, $K_i = 0$ and Eq. (9) becomes a quadratic algebraic equation. Its solution was treated in the recent publication by Cameron et al. (1994). In more general case of $K_i \neq 0$, inserting Eq. (8) into Eq. (9) leads to an algebraic equation of the 3rd degree, which could be solved analytically or numerically.

Parametrization

To perform a numerical solution of the model described above, the parameters involved into equations must be pre-estimated. The permeabilities for the LDPE film were estimated independently by the concentration-increase method as $p_1 = 0.46 \times 10^{-8}\ m^2\ hr^{-1}$ and $p_2 = 2.0 \times 10^{-8}\ m^2\ hr^{-1}$.

The Michaelis-Menten parameters v_m, K_M and K_i were estimated from observations on the fruit respiration in a closed system where $P_1 = P_2 = 0$. As shown above, $C_2 = Q - C_1$, with Q being constant in the closed system and equals to $Q_A = C_{1A} + C_{2A}$. Combining this equation with Eq. (1) and (3) gives

$$dC_1/dt = -v_m C_1\ 100W/(K_M + C_1 + K_iC_1(Q - C_1))/V \qquad (10)$$

Integration of this differential equation leads to the following non-linear relationship

$$Ut = [K_M\ ln\ (C_{1A}/C_1) + (1 + Q_AK_i) \qquad (11)$$
$$(C_{1A} - C_1) - K_i(C_{1A}^2 - C_1^2)/2]/v_m$$

where U is the following combination of constants of the system

under observation

$$U = 100W/V \qquad (12)$$

The time course of gas concentrations depends not only on respiration parameters but also on the free volume of the system and fruit weight involved as a combination given by Eq. (12). Time multiplied by the ratio U was used here instead of t to bring together the experimental data obtained with the packages containing fruits of different weights and volumes. As can be seen from Eq. (11), the recalculated variable Ut is a nonlinear function of C_1 containing only the Michaelis-Menten parameters, which are to be estimated. For the case of $K_i = 0$, Eq. (11) becomes

$$Ut = [K_M\ ln\ (C_{1A}/C_1) + (C_{1A} - C_1)]/v_m \qquad (13)$$

To estimate v_m and K_M, CO_2-sorbing materials were added into the hermetically closed jar to prevent inhibition of respiratory activity by accumulated CO_2. In this case $K_i = 0$. The time course of oxygen depletion observed in the closed system with CO_2-absorber was treated by Eq. (13). The results of these experiments are represented (Fig. 4) by triangles. The parameters estimated by nonlinear curve fit are: $v_m = 38.4 \times 10^{-6}\ m^3/kg/h$ and $K_M = 32.56\%$. The lower curve (Fig. 4) is the result of the non linear fitting to the data from experiments with CO_2-sorbing materials.

The next step was to analyze the closed system experiments without CO_2 sorbent. The observations are represented (Fig. 4) by circles. Eq. (11) was used to estimate the parameter of inhibition, K_i, taking the values of the parameters v_m and K_M as found above. The estimated value of the parameter is $K_i = (0.1259 \pm 0.0188)\ (\%)^{-1}$. A comparison of the fitted curve (the upper one) was made with the observed points (Fig. 4). Use of the variable Ut allows to compare experimental data obtained with packages containing fruits of different weights and volumes. The collection of data from several experiments leads to a distribution of experimental points and may to some extent be explained by the product-to-product variation in O_2 uptake, which was analyzed by Talasila et al. (1994). The influence of inhibition can be seen (comparing lower and upper curves). The equation describing respiration rate was taken, following Lee et al. (1991), in the form of Michaelis-Menten kinetics with non-competitive inhibition. More experimental data are needed to draw conclusions on type and quantitative characteristics of inhibition.

The parameters of Michaelis-Menten kinetics for oxygen uptake by bulky plant organs may vary for different objects and differ widely from those for cytochrome oxidase activity, as discussed by Forward (1965). Note that Michaelis-Menten equations may describe either the rate of enzymatic reaction, or the flux through one of the tissue barriers, especially if an active transport takes place, as discussed by Nobel (1974). According to Chevillotte (1973) and Tucker and Laties (1985), before reaching the enzyme, oxygen is slowed by diffusion. They concluded, that the most obvious effect of diffusion barriers was an increase in apparent K_M for oxygen of any given oxidase, and this disturbance was a function of size and morphology of the commodity. Joles et al. (1994) suggested to substitute the standard notation K_M for $K_{1/2}$ because it is an apparent value. They found $K_{1/2} = 5.6\ kPa$ for red raspberry fruit.

Validation of the model

Simulation of MA-package of bell pepper. As a model for fruits packed by semi-permeable film, Eqs. (1) and (2) are used with Michaelis-Menten type Eq. (3) instead of functions $r_1(C_1, C_2)$ and $r_2(C_1, C_2)$. The parameters involved in Eq. (3) are estimated from the previous independent experiments. Using these Michaelis-Menten parameters, the bell pepper respiration rate was calculated for open air conditions as $\approx 14.5 \times 10^{-6}\ m^3/kg/hr$, which coincides with 13–14 mL/kg/hr measured for
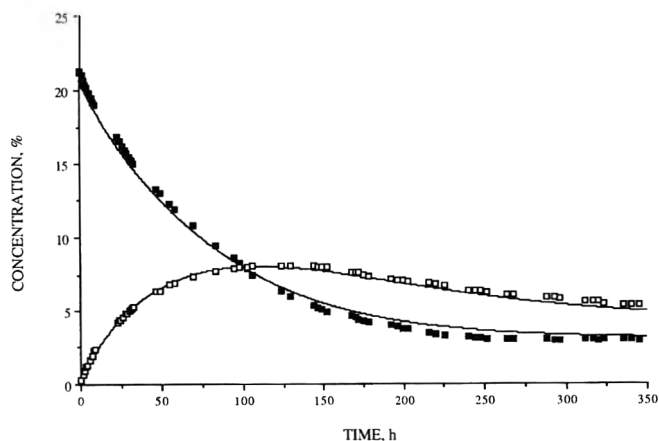
Fig. 5—The model validation. The curves represent time course of oxygen and carbon dioxide concentrations predicted by the model. The points represent the experimental data.

nonpacked Maor pepper by Lurie et al. (1986). This is an additional independent checking of validity. The MA-package model was validated by comparison of its predictions with observations performed on pepper fruit placed in the chamber closed with the LDPE film.

Results of the dynamic simulation (curves) were compared (Fig. 5) with the experimental results (points) for the time course of O_2 and CO_2 concentrations. The observations were well correlated with time and do not reveal a distinct distribution. They result from one prolonged experiment with the same commodity in the same MA system and with automatically measured gas concentrations. The predicted gas dynamics is in good agreement with the observed time course during long-term experiments. The steady-state concentrations of O_2 and CO_2 predicted for this system using Eq. (8) and (9) are $C_1 = 3.0\%$ and $C_2 = 4.15\%$, respectively. The experimentally obtained concentrations approach the calculated steady-state values.

The predicted maximum on the $C_2(t)$ curve fits the recorded one and is about 8%, which markedly exceeds the steady-state value (4.15%) during a rather long time period. The maximum on the curve for CO_2 kinetics (overshooting effect) could be seen in the data presented by many workers (Tomkins, 1962; Jurin and Karel, 1963; Hayakawa et al., 1975; Geeson et al., 1985), but it was not considered as an intrinsic characteristic of the system dependent on its parameters. Consequently, estimation of optimal conditions for fruit storage based on analysis of steady-state concentrations may not be enough for such commodities as pepper, apples, tomatoes, etc. The study of the transient period, as performed here, is important, because the produce may spend a prolonged part of storage under transient conditions.

Predictions of the model

After being validated, the model can be utilized to study other forms of MA packages by means of computer simulations instead of conducting long-lasting and expensive experiment. The model allows to change design parameters of the package (such as free volume, film thickness, area of film, etc.) and to compute how such changes would affect gas dynamics and steady-state gas levels.

Response of the system to variation of design parameters may be predicted to some extent from the general Eq. (1) and (2). The free volume of the package atmosphere (V), enters these equations in a way which allows to combine it with time by multiplying both sides by V and replacing t by a new variable $\tau = t/V$. Changing V is equivalent to rescaling the time variable. Another combination is built from parameters A and ΔX. They enter the general equations only as a ratio $A/\Delta X$. This means
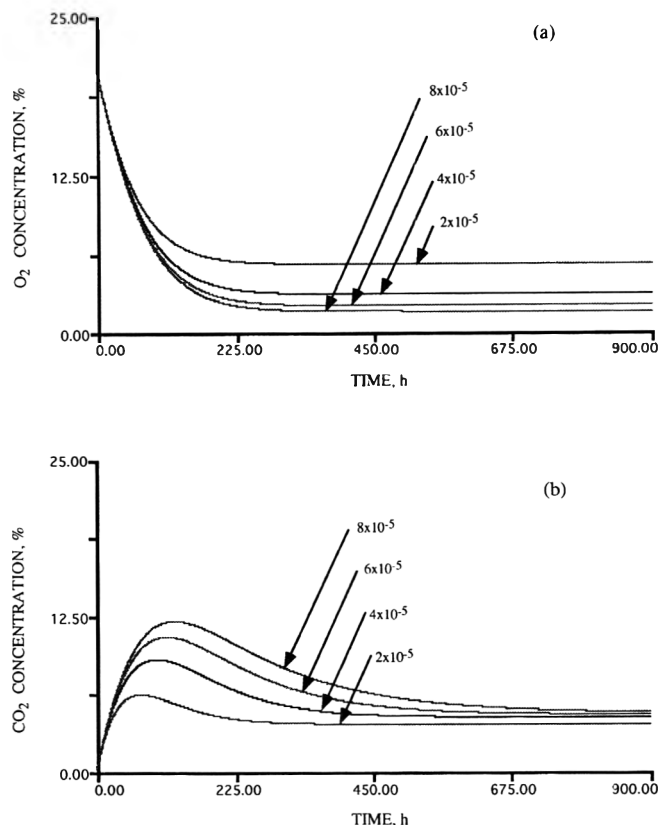
Fig. 6—The model predictions for films of different thickness, ΔX = (2 × 10⁻⁵, 4 × 10⁻⁵, 6 × 10⁻⁵ and 8 × 10⁻⁵) m: (a)—time course of O_2 concentration; (b)—time course of CO_2 concentration.

that increase of A is equivalent to decrease of ΔX and vice versa. The response of the model predictions to variations of these parameters was studied by computer simulations.

As a first example, the thickness of LDPE film (ΔX) was varied. The model was run for $\Delta X = 2 \times 10^{-5}, 4 \times 10^{-5}, 6 \times 10^{-5}$ and 8×10^{-5} m. Figure 6a shows the model prediction for the dynamics of O_2 concentration with these four values of ΔX. The equilibrium values of O_2 were reached at about 300 hr for all four films. The time needed to approach the steady-state of CO_2 is markedly longer and depends on film thickness: the thicker the film, the longer the transient period and the higher the overshooting (Fig. 6b). To compare the changes resulted from A and ΔX variations, the model was run for A = 0.02, 0.03, 0.04 and 0.05 m². The influence of area variation is reciprocal to the effect of changes in film thickness, as predicted above. In this case, the larger the area, the shorter the transient period and lower the maximum on the CO_2 concentration curve.

Henceforth, sensitivity of the system to parameter V was studied (Fig. 7) and represents results of the model runs with V = 0.00124, 0.001, 0.00075 and 0.0005 m³. The time course of O_2 and CO_2 concentrations (Fig. 7a, 7b) shows that variation of V changes the time scale and does not affect the steady-state level of concentrations, nor the extent of overshooting in the CO_2 dynamics. Diminishing V "deforms" the curves along the time axis making shorter the interval needed to approach steady-state or CO_2 maximum.

The experimental system we used to validate the model had much free volume compared to the respiring biomass. As a result, the time needed to reach steady-state in this case was much longer than that reported in many other works. Running the model simulations with different sets of parameters, the film specifications and package dimensions can be found, which would provide the best conditions for storage of a given commodity.
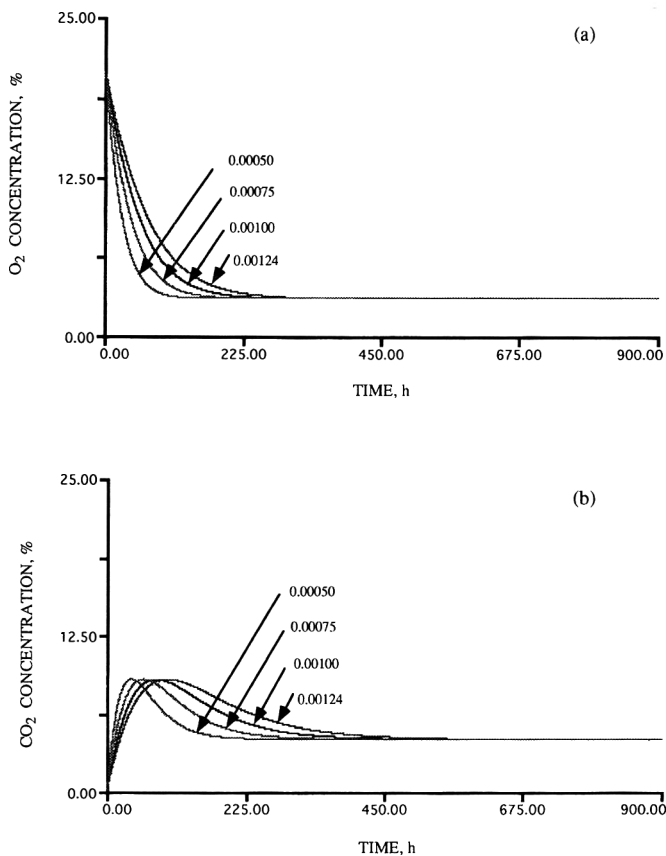
Fig. 7—The model predictions for packages of different free volumes, V = (0.00124, 0.001, 0.00075 and 0.0005) m³: (a)—time course of O₂ concentration; (b)—time course of CO₂ concentration.

CONCLUSIONS

THE MATHEMATICAL ANALYSIS of the MA package model showed that the time course of CO_2 concentration had a maximum if film permeability to CO_2 is greater than that to O_2. Since most of the plastic films have $p_2 > p_1$, the maximum on CO_2 curve is an intrinsic feature of the system. This means that during a part of the transient period, the concentration of CO_2 in the system may be markedly greater than the evaluated steady-state value. This must be considered when optimal conditions for MA packaging of fruits and vegetables are under investigation. The length of transient period may be changed by variation of free volume of the package, as shown by sensitivity analysis. The sum of the O_2 and CO_2 concentrations, Q, may be utilized to indicate a change of RQ in the system. Equations for prediction of the steady-state concentrations were suggested for the case when the enzyme kinetics includes the inhibition process. The parameters of the respiration process were estimated by independent experiments in a closed system. The model validated with these parameters could be used for choosing film specifications and package dimensions, which would provide a desired composition of atmosphere in the MA package.

NOMENCLATURE

Symbol	Description	Dimension
A	Surface area of the film	m^2
C_1	Concentration of O_2 in the package	%
C_2	Concentration of CO_2 in the package	%
$\mathbb{C}_1$	Steady-state value of C_1	%
$\mathbb{C}_2$	Steady-state value of C_2	%
C_{1A}	Concentration of O_2 in the outside atmosphere	%

C_{2A}	Concentration of CO_2 in the outside atmosphere	%
K_i	Coefficient of inhibition	$(\%)^{-1}$
K_M	Michaelis-Menten constant	%
p_1	Coefficient of film permeability to O_2	m^2hr^{-1}
p_2	Coefficient of film permeability to CO_2	m^2hr^{-1}
P_1	Characteristic of system's permeability to O_2	hr^{-1}
P_2	Characteristic of system's permeability to CO_2	hr^{-1}
Q	Sum of O_2 and CO_2 concentrations	%
$r_1(C_1,C_2)$	Rate of O_2 consumption due to respiration	$m^3kg^{-1}hr^{-1}$
$r_2(C_1,C_2)$	Rate of CO_2 evolution due to respiration	$m^3kg^{-1}hr^{-1}$
v_M	Michaelis-Menten maximum rate	$m^3kg^{-1}hr^{-1}$
V	Free volume of the package	m^3
W	Weight of the fruits in the package	kg
ΔX	Thickness of the packaging film	m

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Quality of Electron Beam Irradiated Strawberries

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ABSTRACT

Fresh 'Tristar' strawberries were treated by electron beam irradiation to determine the effects on postharvest quality attributes and shelf life. The intensity of red color rated by sensory panelists decreased as irradiation dosage increased from 0 to 2 kGy. Hunter 'L' values were higher for fruit treated with 2 kGy than for 0 and 0.5 kGy. Instron firmness values were lower for all irradiated fruit than for control fruit. Panelists rated irradiated fruit less firm than nonirradiated fruit stored 1, 2 and 4 days. An increase in off-flavor was noted among all treatments stored 6 and 8 days. Irradiation suppressed fungi on stored berries. Irradiation doses of 1 and 2 kGy extended shelf life 2 and 4 days, respectively. Electron beam irradiation technology has excellent potential for extension of shelf life of fresh strawberry fruits.

Key Words: electron irradiation, color, strawberries, firmness, storage stability

INTRODUCTION

IRRADIATION is an effective method for reducing food spoilage organisms and for increasing shelf life (IFT, 1983; WHO, 1988). Strawberry quality is reduced primarily by infection with fungi such as *Botrytis cinerea* and *Rhizopus stolonifer* after harvest. Irradiation of strawberries at doses not to exceed 1 kGy was approved by the U.S. Food and Drug Administration (FDA, 1986). Strawberries, treated by gamma irradiation, were first marketed in the United States in January 1992 (Marcotte, 1992). That success suggested potentially increased markets for irradiated strawberries.

Previous research and market tests of strawberries were conducted with gamma rays as irradiation sources. There is no research on electron beam irradiation of strawberries, or other fruits, vegetables, or plant-based foods. The benefits of electron beam irradiation are that it does not require a radioactive source and the irradiation may be accomplished at a faster dose-rate than gamma rays. Electron beam irradiation provides the possibility of integrating treatment into the product line and may have an economic advantage over gamma rays when thickness of the product is small and quantity of product is large (Loaharanu, 1994). Electron beams penetrate 4–5 cm so it would be necessary to irradiate strawberries in flats which could be palletted after treatment.

Strawberries may tolerate gamma irradiation up to 2 kGy (Thomas, 1986). Doses in excess of 2 kGy caused texture and color changes of strawberries (Thomas, 1986). Zegota (1988) noted the loss of color of strawberries after treatment with 2.5 kGy and 3.0 kGy irradiation. Johnson et al. (1965) reported that strawberries became softer as irradiation dose increased from 1 kGy to 4 kGy.

Our objective was to determine the effects of electron beam irradiation on chemical and physical characteristics, sensory qualities, and fungal colonization of strawberries. A secondary objective was to determine the feasibility of electron beam irradiation treatment as a means for extending shelf life of fresh strawberry fruits.

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MATERIALS & METHODS

'TRISTAR' STRAWBERRIES (*Fragaria* × *ananassa* Duch.) were harvested at the Iowa State University Horticulture Research Farm, Ames, IA, transported to a laboratory within 1 hr after harvest. and sorted for size (2.0 to 2.5 cm diam) and degree of ripeness (fully red ripe, with no blemishes) into plastic containers (20.6 × 15.0 × 4.0 cm) with air-exchange holes. About 35 berries were placed one layer deep in each container. Three containers/treatment were prepared. Fruit were not washed before irradiation. All treatments were stored at 2°C ≈ 3 hr before irradiation.

Fruit in the containers were irradiated with 0-, 0.5-, 1- and 2-kGy doses of electron beam irradiation at an energy level of 10 MeV at the Linear Accelerator Facility (LAF), Iowa State University, Ames, IA. Procedures for irradiation treatment and handling of strawberries were determined in preliminary testing in consultation with technicians at the LAF. All treatments were transported in closed cardboard boxes to the LAF. Transportation required 15 min. Irradiation treatment required about 30 min; the temperature of the LAF was 20°C. Fruit was refrigerated at the LAF until irradiation treatment. Samples were stored in the same cardboard boxes at a relative humidity of 40–60%.

Each treatment consisted of a combination of irradiation dosage, storage temperature (2°C or 21°C) and storage time (0, 1, 2, 4, 6, 8 days at 2°C or 0, 1, 2, 3 days at 21°C). Three replications (harvest dates September 13, 16 and 20, 1993) of all treatments were completed.

Sensory evaluation

Strawberries stored at 2°C were evaluated by an 11-member trained sensory panel 0, 1, 2, 4, 6 and 8 days after irradiation. Panelists were selected from staff and students and trained in 4 1-hr sessions. A 15-cm line scale was used to evaluate color, seediness, firmness, juiciness, sourness. flavor, and off-flavor. Green fruit, seeds not prominent, mushy, extremely dry, no sourness, no fruit flavor and no off-flavor were scored 0; brown-red fruit, extremely prominent seeds, extremely firm, extremely juicy, extremely intense sourness, extremely intense fruit flavor and extremely intense off-flavor were scored 15. Panelists were presented fruit with off-flavors (fermented, moldy, musty, etc.) during training and were instructed to identify specific off-flavors, if any, in samples.

Two randomly selected berries of each treatment (4 doses) were placed into individual cups coded with random 3-digit numbers. Panelists evaluated samples in individual booths and were instructed to rinse their mouths with water and to eat unsalted crackers between samples. Procedures for evaluation of strawberries followed those described by Reitmeier and Nonnecke (1991).

The effects of irradiation dose (4) and storage time (6) on sensory characteristics of strawberries stored at 2°C were analyzed in a split plot design by analysis of variance (SAS Institute Inc., 1989). Panelists did not evaluate fruit after 4 days storage for 0 and 0.5 kGy-treated samples and after 6 days for 1 kGy-treated fruit due to moldy, unacceptable fruit. Main effect means were reported for color, seediness, juiciness, sourness, flavor and off-flavor. There was an interaction between dose and storage time for firmness so interaction means were reported. When F-values were significant, least significant differences (LSD) at p ≤ 5% were calculated.

Chemical analysis

Percentage titratable acidity (TA), pH, and soluble solids concentration (SSC) measurements were completed for all treatments in duplicate. Berries (≈100g) from each treatment were decapped and blended for 30 sec in a blender. Puree (5g) was diluted to 100 mL with deionized water, titrated to pH 7.0 with 0.1N NaOH, and reported as percentage of citric acid. The pH of the puree was measured with a Fisher (Model 640A) pH meter. Soluble solids concentration (SSC) was determined by extruding a sample of puree through 2 layers of tissue onto the lens of a Reichert-Jung refractometer.

Physical analysis

Color was determined with a Hunter Tristimulus Colorimeter (Model D-25, Hunter Laboratories, Reston, VA) standardized with a red tile (no. C20-2224, L = 34.2, a = 50.2, b = 20.9). Puree (≈40 mL) was poured into a standard glass container, and L, a and b measurements of puree were recorded. Hue angle (tan^{-1}b/a) and saturation index [(a^2 + b^2)$^{1/2}$] were calculated (Clydesdale, 1978; Setser, 1984).

Texture was determined by using an Instron Universal Testing Machine (UTM, Model 1122, Instron Corp., Canton, MA). Ten ripe berries (2- to 3-cm diameter) of each treatment were used. A star-shaped (5-point, 1-cm-diam) probe was used to penetrate the side of the berry with the stem in a horizontal plane at a crosshead speed of 500 mm-min^{-1} and a chart speed of 200 mm-min^{-1} (Ourecky and Bourne, 1963). The amount of force to break the skin and puncture the core was expressed in Newtons. A mean of 10 berries was used to determine firmness of each treatment.

Fungal colonization

Subsamples of three berries each were added to 100 mL of sterile deionized water (SDW) in a 250-mL beaker and shaken for 1 hr on a reciprocal shaker at 140 rpm (Gleason et al., 1991). After making serial, 10-fold dilutions of the extract in SDW, 0.1-mL aliquots of each dilution were plated on acidified potato-dextrose agar (APDA). Culture plates were incubated on a laboratory bench at 21–25°C. At 24 and 48 hr after plating, the total number of fungal colonies growing on each plate was counted using a dissecting microscope at 30× magnification.

To determine composition of the fungal microflora, colonies were subsampled from APDA plates derived from berries that had been irradiated at 0, 0.5, 1 or 2 kGy, then incubated for 2 days at 21°C before extracting. Fifteen arbitrarily chosen colonies were transferred from each plate, then incubated on a laboratory bench at 21–24°C until they could be identified by species or genus based on morphology of the mycelium and spores.

Statistical analysis

The data were analyzed based on a randomized complete block design. Analysis of variance (SAS Institute, Inc., 1989) was used to analyze effects of irradiation dose and storage time. When a significant interaction between irradiation dose and storage time resulted, interaction means were reported. Main effect means were reported when no significant interactions were found. When F-values were significant, least significant differences (LSD) at p ≤ 5% were calculated.

RESULTS & DISCUSSION

EFFECTS OF IRRADIATION (Table 1) and storage time (Table 2) on the chemical and physical properties of strawberries stored at 2°C were compared. Weight loss of fruit stored at 2°C was not influenced by irradiation or storage. There were no differences in pH, SSC and TA values between irradiated and non-irradiated strawberries. The pH of the fruit rose after 8 days storage. Zegota (1988) also reported no change in strawberry pH at the beginning of storage, and a slight decrease in total acidity after 2 wks of 2-4°C storage. The titratable acidity decreased after 2 days storage (Table 2). Couture et al. (1990) found no change in titratable acidity of strawberries during storage or after irradiation. Beyers et al. (1979) and Chachin et al. (1968) also reported no change in sugar content of strawberries after gamma irradiation.

Instron firmness values showed that strawberries were less firm after irradiation and increased slightly in firmness during storage. Johnson et al. (1965) also reported an immediate loss in flesh firmness of strawberries following irradiation and firmness increased during cold storage. Both skin and flesh firmness values were lower after 8 days of 2°C storage. The loss of firmness after irradiation may be due to changes in pectin and cellulose content (Kertesz et al., 1964; Chachin et al., 1968; Belli-Donini and Stornaiuolo, 1969; d'Amour et al., 1993). Water loss during storage was not great enough to account for the increasing firmness in storage, but it may have been due to reabsorption of water from intercellular spaces into the cell. The reabsorption of calcium from the cell sap followed by calcium

bonding in the pectin may contribute to increasing firmness during cold storage (Maxie and Abdel-Kader, 1966).

Hunter 'L' values were higher for fruit treated with 2 kGy than controls and 0.5 kGy-treated fruit. No differences were noted in Hunter 'a' and 'b', hue angle, and saturation index values among all irradiation doses. Zegota (1988) reported loss in redness after irradiation and storage. Couture et al. (1990) indicated that irradiation at 0.3 kGy slightly delayed color development in the fruit, while Truelsen (1960) found no effect on red color of strawberries after irradiation with gamma rays at a dose of 2 kGy.

Sensory color values decreased (to more green and lighter red) as irradiation dose increased (Table 3), which coincided with higher 'L' values. Strawberries treated with 0.5-kGy and 2-kGy doses of irradiation were more juicy than nonirradiated fruit. No differences among doses were noted in fruit seediness, sourness, flavor or off-flavor intensity.

Fruit color became more brown-red during storage (Table 4). No changes were found in seediness, juiciness, sourness or flavor during storage. After 6 days storage at 2°C, the off-flavor intensity of strawberries increased. Panelists noted moldy, musty and fermented types of off-flavors.

Sensory firmness values (Table 5) were similar to the Instron UTM results. Firmness tended to decrease as irradiation doses increased, although loss of firmness immediately after irradiation was not indicated. Sensory panelists rated fruit irradiated with 2 kGy dose less firm than nonirradiated fruit stored 0, 1, 2 and 4 days. After 24 hr storage at 2°C, firmness increased for the control and 0.5-kGy treated strawberries possibly due to effects of cooling. As expected, firmness generally decreased as storage time increased.

Effect of irradiation and storage time (0, 1, 2, 3 days) on pH, SSC, TA, texture and color of strawberries stored at 21°C were similar to results of storage at 2°C (data not shown). Irradiation treatment at all doses decreased weight loss of strawberries stored at 21°C, while weight loss increased as storage time increased. The storage temperature of 21°C was used to determine whether irradiation could result in no need for refrigeration for fruit storage. Fruit were moldy in 3 days at 21°C. Thus, reduction of temperature was crucial in maintenance of strawberry quality.

The shelf life of strawberries was extended as irradiation dose increased. After 4 days of 2°C storage, fungal mycelia were visible in nonirradiated samples, while 0.5 kGy and 1 kGy-treated fruit revealed visible mycelia at 6 days and 2 kGy-treated fruit revealed visible mycelia at 8 days. Zegota (1988) reported that nonirradiated strawberries were visibly infected by mold after 1 wk cold storage, while 2.5 and 3.0 kGy-treated fruit began to mold at 2 wk.

Irradiation suppressed proliferation of fungi on berries (Fig. 1). Under both storage temperatures, the number of colonies recovered (y, converted to log$_{10}$ scale) increased as storage time increased, but dose × storage time interactions were not significant (data not shown). Data was averaged over 3 replications and all storage times. Orthogonal comparisons showed that the number of colonies recovered from nonirradiated samples was greater than numbers for irradiated treatments (prob > F ≤ 0.05). At 2°C, reduction in number of fungal colonies in response to increasing irradiation level (x) was described by the equation y = 4.08 − 0.76X (MSE = 1.00, F$_{1,6 df}$ = 17.74, prob > F = 0.0056). At 21°C, the relationship was described by the equation y = 4.04 − 0.64X (MSE = 0.229, F$_{1,6 df}$ = 46.97, prob > F = 0.0005). Strawberry shelf life was extended as irradiation dose increased.

The fungus that was isolated most frequently was *Collectotrichum acutatum*, the causal agent of anthracnose fruit rot. This result was somewhat expected, because a severe epidemic of the disease occurred in the field during the sampling period. Although none of the berries sampled had lesions when sampled, inoculum of the pathogen would have been distributed through-

Table 1—Effect of irradiation on strawberries stored 0-8 days at 2°C

(KGy) Dose	Weight loss (%)	pH	(%) SSC	(%) TA	Firmness (N) Skin	Flesh	L	a	b	HAª	SIᵇ
0	0.36	3.23	8.47	1.16	4.56ᶜ	7.01ᶜ	24.2ᵈ	35.2	12.5	19.5	37.3
0.5	0.38	3.26	8.29	1.18	3.97ᵈ	6.04ᵈ	24.2ᵈ	35.1	12.3	19.3	37.2
1	0.57	3.25	8.46	1.14	3.98ᵈ	5.93ᵈ	24.4ᶜᵈ	35.0	12.3	19.4	37.1
2	0.52	3.29	8.56	1.15	3.69ᵈ	5.35ᵉ	25.0ᶜ	34.8	12.4	19.6	36.9
	NS	NS	NS	NS				NS	NS	NS	NS

ª HA = hue angle (tan⁻¹ b/a).
ᵇ SI = saturation index $[(a^2 - b^2)^{1/2}]$.
ᶜ⁻ᵉ Means of three replications and six storage times. Means within columns followed by the same superscript are not significantly different ($p < 0.05$); NS = not significant.

Table 2—Effect of storage time on irradiated strawberries stored at 2°C

Days	Weight loss (%)	pH	(%) SSC	(%) TA	Firmness (N) Skin	Flesh	L	a	b	HA	SI
0	0	3.21ᵇ	8.75	1.24ª	3.74ᵇᶜ	5.98ª	26.8ª	35.9ª	13.7ª	20.8ª	38.4ª
1	0.38	3.24ᵇ	8.51	1.20ª	4.26ª	6.28ª	23.9ᵇᶜ	34.4ᵈᶜ	12.1ᶜ	19.4ᵇᶜ	36.4ᵇᶜ
2	0.30	3.21ᵇ	8.36	1.14ᵇ	4.02ªᵇᶜ	6.33ª	24.7ᵇ	35.8ªᵇ	12.8ᵇ	19.7ᵇ	38.0ª
4	0.51	3.27ᵇ	8.26	1.13ᵇ	4.21ªᵇ	6.10ª	24.0ᵇᶜ	34.9ᵇᶜ	12.1ᶜ	19.1ᵇᶜ	37.0ᵇ
6	0.71	3.27ᵇ	8.27	1.09ᵇ	4.15ªᵇ	5.97ª	23.5ᶜ	34.8ᶜᵈ	11.8ᶜ	18.8ᶜᵈ	36.7ᵇᶜ
8	1.17	3.41ª	8.48	1.10ᵇ	3.60ᶜ	5.29ᵇ	23.0ᶜ	34.0ᵈ	11.2ᵈ	18.3ᵈ	35.9ᶜ
	NS			NS							

ª⁻ᵈ Means of three replications and four irradiation doses. Means within columns followed by the same superscript are not significantly different ($p < 0.05$); NS = not significant.

Table 3—Sensory evaluation of stored strawberries – effect of irradiation dose

KGy	Color	Seed	Juice	Sour	Flavor	Off-flavor
0	11.1ª	5.8	8.6ᵇ	7.0	8.5	1.1
0.5	10.6ᵇ	5.7	9.1ª	7.3	8.5	1.1
1	10.0ᶜ	5.8	8.9ªᵇ	6.8	7.7	1.8
2	9.5ᵈ	6.2	9.4ª	7.4	8.2	1.6
		NS		NS	NS	NS

ª⁻ᵈ Means of three replications, eleven panelists and six storage times. Means within columns followed by the same superscript are not significantly different ($p < 0.01$); NS = not significant. 0 = green, seeds not prominent, extremely dry, no sourness, no strawberry flavor and no off-flavor to 15 = brown-red, extremely prominent seeds, extremely juicy, extremely intense sourness, extremely intense strawberry flavor and extremely intense off-flavor.

Table 4—Sensory evaluation of irradiated strawberries – effect of storage time (2°C)

Daysª	Color	Seed	Juice	Sour	Flavor	Off-flavor
0	9.8ᵈ	5.5	9.1	6.8	8.3	1.4ᶜ
1	9.9ᶜᵈ	5.6	9.2	6.8	8.3	1.1ᶜ
2	10.4ᵇᶜᵈ	5.8	9.0	6.9	8.8	1.2ᶜ
4	10.7ᵇ	6.3	9.1	7.5	8.0	1.2ᶜ
6	10.5ᵇᶜ	6.4	8.5	7.8	7.8	2.7ᵇ
8	10.3ᵇᶜᵈ	6.6	9.1	7.4	7.2	2.7ᵇ
		NS	NS	NS	NS	

ª Fruit evaluated at 0, 1, 2, and 4 days represent 0, 0.5, 1 and 2 kGy-dose treatments. Fruit evaluated at 6 days included 1 and 2 kGy-dose treatments; at 8 days, 2 kGy-dose only.
ᵇ⁻ᵈ Means of three replications, eleven panelists and four irradiation doses. Means within columns followed by the same superscript are not significantly different ($p < 0.01$); NS = not significant. 0 = green, seeds not prominent, extremely dry, no sourness, no strawberry flavor and no off-flavor to 15 = brown-red, extremely prominent seeds, extremely juicy, extremely intense sourness, extremely intense strawberry flavor and extremely intense off-flavor.

Table 5—Sensory firmness of strawberries

Dose (kGy)	0	1	Storage time (days) 2	4	6	8
0	6.6ᵉᶠᵍ	7.5ⁱʲ	7.1ᶠᵍʰⁱ	8.0ʲ	_ᵏ	_ᵏ
0.5	6.7ᵉᶠᵍʰ	7.5ⁱʲ	5.9ᵇᶜᵈ	6.8ᵉᶠᵍ	_ᵏ	_ᵏ
1	7.19ʰⁱ	6.5ᵈᵉᶠ	6.5ᵈᵉᶠ	5.3ª	7.3ʰʲⁱ	_ᵏ
2	6.2ᶜᵈᵉ	6.4ᵈᵉ	5.2ª	6.3ᶜᵈᵉ	5.4ªᵇ	5.7ªᵇᶜ
	6.6	7.0	6.2	6.6	6.2	5.7

ª⁻ʲ Means of three replications and eleven panelists. Means followed by the same superscript are not significantly different ($p < 0.01$); NS = not significant. 0 = mushy to 15 = extremely firm.
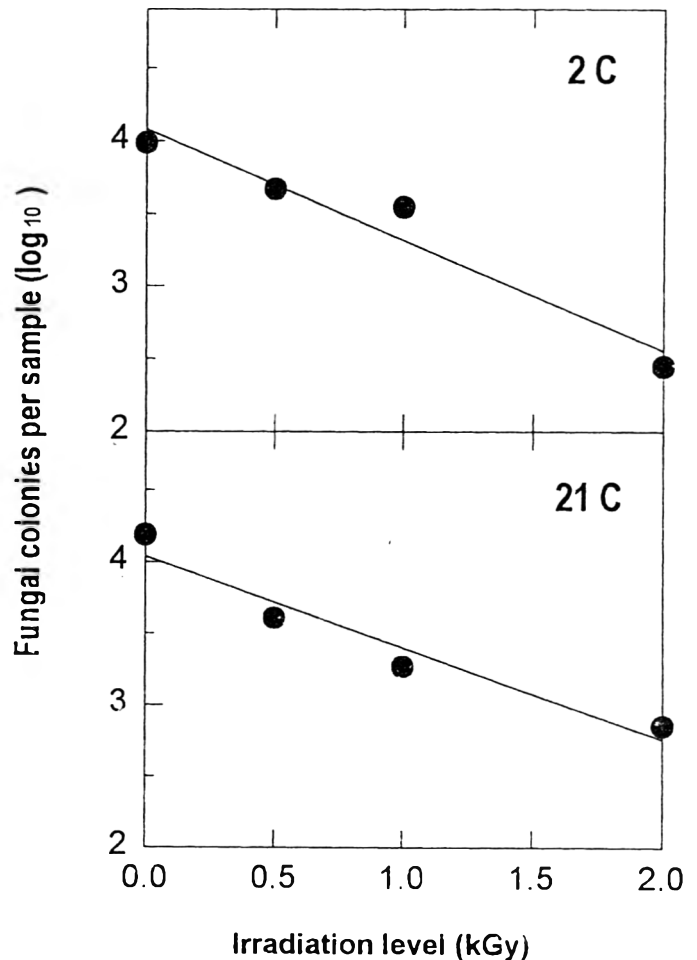ᵏ Moldy fruit not evaluated by sensory panelists.

Fig. 1—Mean number of fungal colonies per berry sample (y) following exposure to 0, 0.5, 1.0, or 2.0 kGy irradiation, as measured by the dilution plating method. Fungal colonization of treated berries stored at 2°C was determined after 1, 2, 4, 6, or 8 days. Berries stored at 21°C were sampled at 0, 1, 2, or 3 days after irradiation. Data shown is averaged over three replications and all storage times. Dose X storage time interactions were not significant (data not shown).

out the field, even on asymptomatic leaves and fruit (Gleason, unpublished data). Other genera of fungi commonly isolated from berry extract includes *Alternaria, Cladosporium, Phoma, Pestalotia, Rhizopus, Mucor, Penicillium, Rhizoctonia,* and *Sclerotium.* The absence of *Botrytis cinerea* from the isolations is noteworthy because this fungus is frequently regarded as one of the most serious pre- and postharvest pathogens of strawberry fruit (Maas, 1984). During the September harvest period, however, air temperatures were well above the optimum for *Botrytis* fruit rot. In addition, weekly sprays of vinclozolin (Ronilan 50 WP), a fungicide with excellent activity against *B. cinerea*, may have suppressed it.

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Free Fatty Acids from Orange Juice Absorption into Laminated Cartons and their Effects on Adhesion

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—————————— ABSTRACT ——————————

Orange juice was stored in polyethylene/aluminum foil laminated cartons at 8°C, room temperature (RT), 35°C, and 55°C for up to 1 yr. Studies were carried out on the formation of 6 major free fatty acids (FFAs) in orange juice and their absorption into packaging materials during storage. Simultaneously adhesion tests on packaging materials were performed. Depending on storage temperature FFA concentrations increased steadily during storage and they were absorbed (in parallel) into the food contact layer of the packaging materials. Delamination between the plastic layer and aluminum foil occurred when a critical absorption level of 4–5 mg/dm² packaging material (corresponding to a level of 10.5–13.2 mg/cm³ or 1.1–1.4% in the inside polymer layer) was exceeded.

Key Words: orange juice, packaging, adhesion, free fatty acids, laminated cartons

INTRODUCTION

CITRUS JUICES are often packaged in paperboard packages with aluminum foil as a barrier layer and low density polyethylene (LDPE) as product contact layer. Interaction between these materials and food, involving absorption of aroma compounds, has been studied. The impact of interaction on package performance has been studied much less. The structural integrity of packages may also be affected by interactions between food and packaging materials which should be minimized. The integrity and barrier performance of multi-layer food packages and the adhesion between layers must be maintained during the shelf life of the food. Delamination would reduce mechanical strength of the package, and thus package integrity, and also the barrier effectiveness. This might lead to oxidation and aroma losses.

Adhesion between aluminum foil and polyethylene in multi-layer flexible packages is preferably achieved by use of an intermediate layer of acid copolymer tie resin. Hjertberg and Lakso (1989) showed that the bonding efficiency of carboxylic groups in acid copolymers, such as poly(ethylene-co-acrylic acid) (EAA) and poly(ethylene-co-methacrylic acid) (EMAA) and ionomers based upon them, was higher than that of ester groups in materials such as poly(ethylene-co-vinyl acetate) (EVA) and poly(ethylene-co-butyl acrylate) (EBA). Ulrén et al. (1990) concluded that the bonding to aluminum foil was achieved via Lewis acid/base interactions. The strength of interfacial interaction related to the acidity/basicity and the concentration of functional groups. This was supported by Finlayson and Lancaster (1991) and Hansen et al. (1992), who showed that the acid/base functionality of the aluminum foil surface determined which adhesion level could be achieved to acid copolymers. The same principle was also applicable to adhesion between LDPE and aluminum foil without tie layers. In that case the polar groups were formed in the LDPE during extrusion coating.

Hester et al. (1990) demonstrated that the choice of tie resin in aluminum foil/tie resin/LDPE structures was very important for the durability of adhesion when reactive type foods are packed. Van den Bossche (1991) showed the importance of the type food, content and type of co-monomer in the tie resin, the thickness of the tie layer, and the storage temperature on the durability of adhesion. Both studies emphasized that a higher acid co-monomer in the tie resin had to be used to avoid delamination when packaging foods such as citrus juices. Neither offered an explanation for the reactive nature of such products.

Olafsson et al. (1993a, b) investigated model solutions of organic acids in foods with respect to delaminating effects. The packaging material structure was aluminum foil/LDPE without an intermediate tie layer. They concluded that non-volatile polar acids such as citric- and lactic acid did not affect adhesion of these structures. Volatile acids, e.g. acetic and propionic acid, showed high delaminating effects. Formation of carboxylic salts between the permeating acid and the aluminum foil accompanied the delamination. However their findings were not confirmed with foods.

Adhesion problems encountered during long term storage of citrus juices in multilayer structures have not been satisfactorily explained. They have generally been attributed to absorption of aroma compounds (causing swelling of the polymer and hence stressing interfacial bonds) or generally to the high acidity of the products. However by far the major part of citrus aroma absorption into multilayer flexible packages with aluminum foil as a barrier layer was shown by Dürr et al. (1981) to occur within the first 2 wk storage when equilibrium was reached. By contrast, delamination occurred earliest after several months at ambient temperatures.

The acidity of citrus juices is mainly due to citric acid, which was proven by Olafsson et al. (1993b) not to affect adhesion in multilayer structures. Beside citric acid, however, citrus juices contain FFAs (Nagy and Nordby, 1970; Kealey and Kinsella, 1973), which were found to initiate delamination in model systems. Our objective was to investigate the formation of FFAs in orange juice and to determine whether absorption/permeation and interaction of FFAs were responsible for the delaminating effects of orange juice on aluminum foil/tie resin/LDPE structures during long term storage.

MATERIALS & METHODS

Orange juice samples and heat-treatment

Commercially available deep frozen orange juice concentrate from Brazil was used. It was delivered in steel drums, thawed and stored at 8°C. The concentrate was diluted to 12° Brix. It was pasteurized at 95°C for 15 sec and cooled rapidly to 15°C using a plate heat exchanger. Intermediate storage and packaging were at ≈15°C. The heat treatment and packaging took place at the Tetra Pak Processing plant in Lund, Sweden.

Packaging material and packaging

The orange juice samples were packaged by a Tetra Brik Aseptic filling machine into 250cc packages. The packaging material was a commercially available laminate:

LDPE/Paper board/LDPE/Alufoil/Ionomer/LDPE
7 μm 6 g/m² 29 g/m²

The packaging material was produced via extrusion coating and extrusion lamination. The 2 layers closest to the food were co-extruded.
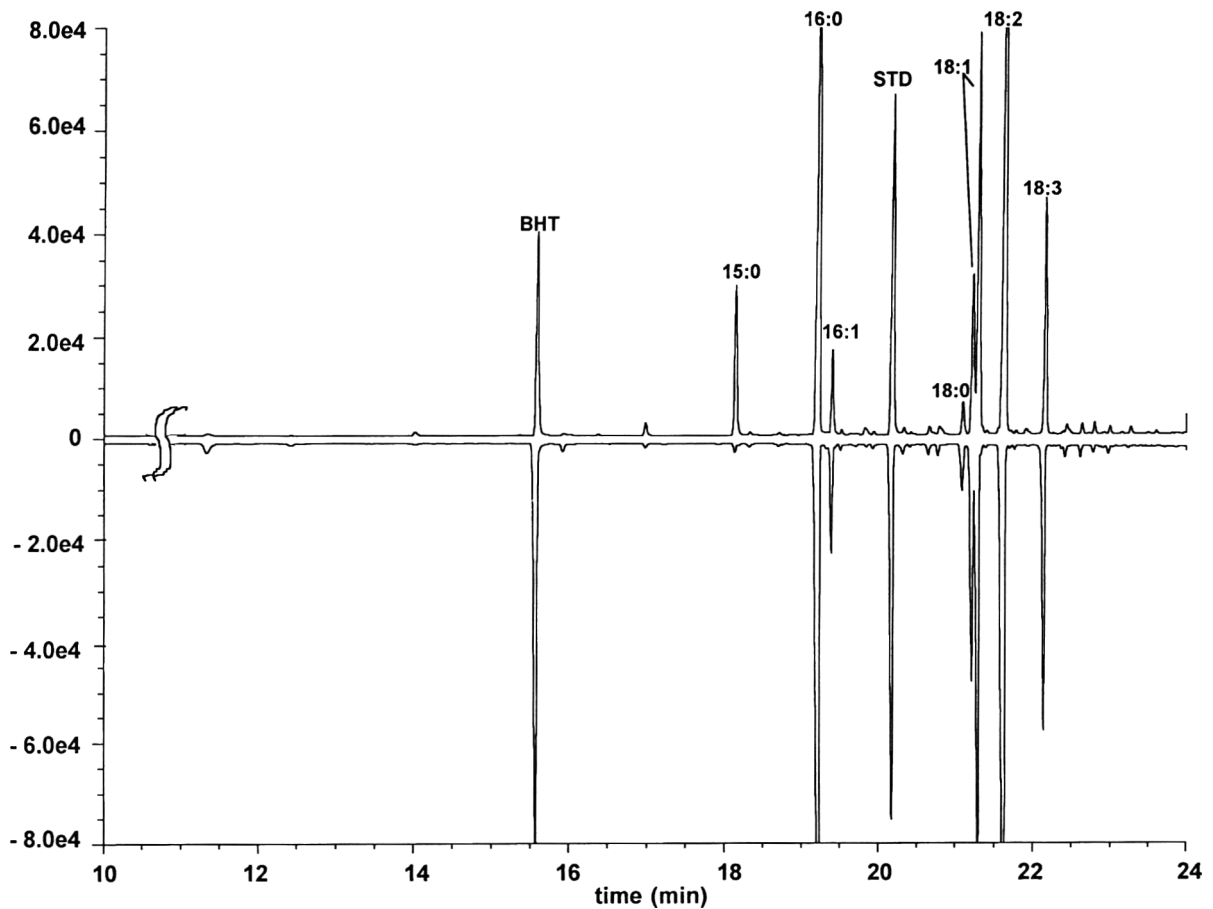
Fig. 1—GC-profile of fatty acid methyl esters from (top) a saponified orange juice extract and (bottom) a packaging material extract. Both samples taken after 12 mo at RT. Peak abbreviations as well as conditions for derivatization and GC-analysis described in Materials & Methods.
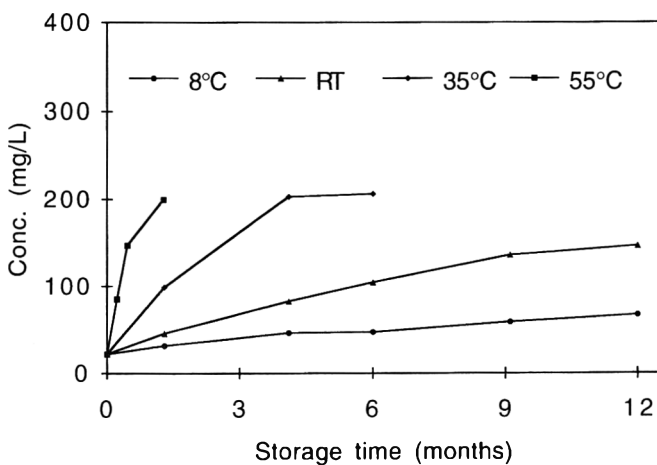

Fig. 2—Changes in concentration of FFAs in orange juice during storage for up to 12 mo at different temperatures.
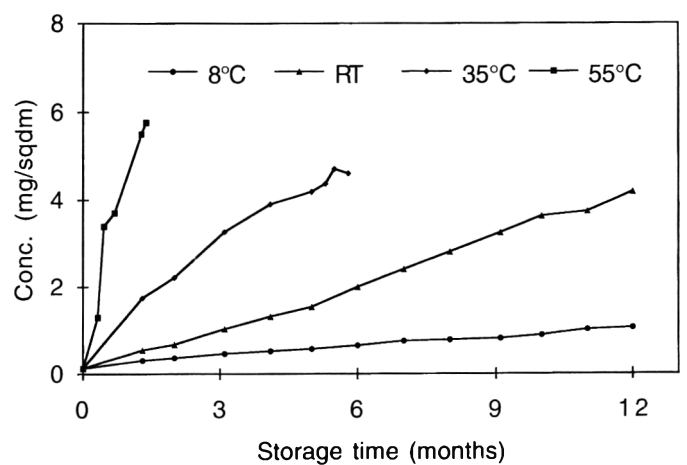

Fig. 3—Changes in concentration of FFAs extracted from the packaging materials during storage for up to 12 mo at different temperatures.

Handling and storage

Samples were kept at Tetra Pak Materials Lund for controlled temperature storage and adhesion measurements and were transported to Tetra Pak GmbH, Stuttgart, Germany for controlled temperature storage and chemical analysis. Samples were stored in both places up to 1 yr at 8°C ± 1°C, RT (≈20–25°C), 35°C ± 1°C, and 55°C ± 2°C during the test period. At each sampling, the orange juice was transferred into glass jars, immediately frozen to −18°C, and stored frozen until the end of the storage test. The analysis of packaging material (FFAs and adhesion) was made immediately after sampling to avoid alterations.

Chemical analysis

All chemicals were of analytical grade and were purchased from commercial sources. All FFAs (GC-standards ≈99%) and fatty acid methyl esters (GC-standards ≈99%) were purchased from Sigma, Deisenhofen, Germany. The BF₃-methanol complex (≈20%) was purchased from Merck/Schuchard, Darmstadt, Germany. Monoglyceride (MG), diglyceride (DG), triglyceride (TG) and phosphatide (PP) standards for the thin layer chromatography (TLC) were supplied by Sigma, Deisenhofen, Germany.
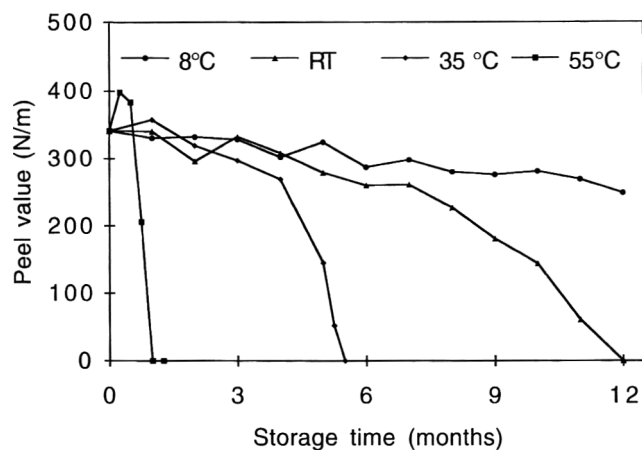
Fig. 4—Adhesion durability in packages during storage for up to 12 mo at different temperatures.

Table 1—Relative weight (%) of FFAs in orange juice and packaging material after storage at room temperature (~20-25°C)

	Storage time (mo)	16:0	16:1	18:0	18:1	18:2	18:3	Sum
				(% of total FFAs)				
OJ[a]	1.3	29.3	2.9	1.3	21.9	33.6	11	45[c]
OJ	12	27.2	2.8	1.0	22.5	37.1	9.5	146[c]
OJ(sap)[c]	1.3	20.7[b]	3.2[b]	1.1[b]	23.3[b]	41.6[b]	10.2[b]	184[d]
OJ(sap)	12	21.9[b]	3.0[b]	1.0[b]	22.0[b]	42.3[b]	9.9[b]	263[d]
Pack.[a]	1.3	28.7	3.3	1.7	21.3	36.9	7.1	0.5[e]
Pack.	12	27.0	3.3	1.7	21.5	39.6	7.1	4.2[e]

[a] Abbrev.: OJ, orange juice extracts; OJ (sap), saponified orange juice extracts, Pack., packaging material extracts.

[b] Relative weight (%) of total fatty acids found in the saponified extracts of the orange juice.

[c] Concentration of FFAs in the orange juice in mg/L.

[d] Concentration of total fatty acids in the saponified orange juice in mg/L.

[e] Concentration of FFAs in the packaging material in mg/dm^2.

Sample preparation

Packaging material. FFAs, which had been absorbed into the inside layers of the packaging materials were extracted immediately after sampling with 100 mL of ether (BHT stabilized) for 3 days at 4 °C. Round-shaped migration cells (contact area of 2 × 0.5 dm^2), for one sided contact were used for extraction. TLC of the packaging material extracts for FFAs, MG, DG, TG and PP identified only FFAs. TG could not be evaluated due to interference with aroma oil. A comparison of the content of fatty acid methyl esters after derivatization with BF3, performed with and without saponification of extracts prior to derivatization, showed no difference in fatty acids content. This indicated that the packaging material extracts contained no fatty acid esters in form of TG and that all fatty acids were present as free acids.

Orange juice. FFAs were extracted from orange juice (50 mL; 0.5 mg pentadecanoic acid in methanol added as internal extraction standard) with five times 50–80 mL of ether (BHT added as antioxidant). The combined ether extracts were dried with sodium sulphate. TLC of the orange juice extracts for FFAs, MG, DG, TG, and PP identified FFAs and to a minor extent DG. PP were only found in trace amounts. TG seemed to be the major fraction, but could not be evaluated free due to interferences with aroma oil. However, saponification of the orange juice extracts prior to derivatization with BF3 gave 1.5 to 7 times higher yields for FFAs compared to an un-saponified extract depending on storage time and temperature. This indicated that esterified fatty acids, most probably TG, must have been extracted to a great extent. The recovery rates of extraction standards for FFAs were 73–110%. All concentrations of FFAs in the juice extracts were calculated to 100% recovery of the internal extraction standard. Duplicate analysis did not vary more than 5% in the total FFA's content.

Preparation for GC-analysis

Packaging material extracts. Aliquots of ether extracts (depending on expected amounts of FFAs) were evaporated to dryness with a rotary evaporator. The dried extracts were dissolved in methanol (50 ppm heptadecanoic acid methyl ester added as internal standard) and FFAs were subjected to methylation with BF3 according to the method described by Wijngarten (1967) with slight modifications. The derivatization was performed in methanol without sodium hydroxide and petroleum ether was used to extract fatty acid methyl esters.

Orange juice extracts. Aliquots of ether extracts (depending on expected amounts of FFAs) were evaporated to dryness with a rotary evaporator. The dried extracts were dissolved in petroleum ether (50 ppm heptadecanoic acid methyl ester added as internal standard) and were used directly for GC-analysis without derivatization to avoid transesterification of fatty acid esters. Other aliquots of dried extracts were dissolved in 0.5N methanolic sodium hydroxide (50 ppm heptadecanoic acid methyl ester added as internal standard). The fatty acids were subjected to methylation with BF3 according to the method described by Wijngarten (1967) with slight modifications to determine the total fatty acids content.

Preparation of specimens for peel test

Specimens (15 mm wide) were cut cross the extrusion direction. Five specimens were removed from each package at each sampling. They were not allowed to dry before measurement. The separation between inside layers and the aluminum foil was started manually, just before measurement.

Analytical techniques

Thin layer chromatography. The packaging material extracts and orange juice extracts were evaluated for contents of MG, DG, TG and FFAs on Kieselgel 60 F254 (Merck, Darmstadt, Germany) with petroleum ether/ether/acetic acid (50 mL/50 mL/1 mL) as mobile phase. Detection was performed in an iodine chamber.

The packaging material extracts and the orange juice extracts were evaluated for PP on Kieselgel 60 F254 (Merck, Darmstadt, Germany) with dichloromethane/methanol/water (65 mL/25 mL/ 4mL) as mobile phase. Compounds were detected with a specific phospholipid spray of Dittmer and Lester (1964).

GC-analysis. The extracts from the orange juice (underivatized FFAs and fatty acid methyl esters) and those from packaging materials were separated by GC (Hewlett-Packard 5890 Series II, on-column injector and FID-detector) on a fused silica capillary column: FFAP-CB (Chrompack GmbH, Frankfurt, Germany) 25m × 0.32 mm i.d. × 0.3 μm film thickness with He as carrier gas. A deactivated fused silica capillary of ≈3m was used as retention gap to improve column life time. The temperature program was 50 100°C at 4°C/min, and from 100–250°C at 10°C/min with holding times of 10 min at 240°C and 5 min at 250°C. The injector was 53°C with the oven track modus on. The detector was 300°C. Compound identification was done by comparison with commercially available fatty acids or fatty acid methyl esters. The following fatty acids were identified: Palmitic acid (16:0), palmitoleic acid (16:1), stearic acid (18:0), oleic acid (18:1), linoleic acid (18:2), linolenic acid (18:3).

Chromatograms of fatty acid methyl esters derived from an orange juice extract were compared to fatty acid methyl esters derived from packaging material extracts (Fig. 1). The unresolved peaks at 21.3 min were additionally investigated on a coupled system of GC infrared detector with mass-selective detector (Hewlett-Packard, Boeblingen, Germany) under similar chromatographic conditions. Both peaks showed almost identical IR- and mass-spectra, in good accordance with the IR- and mass-spectra of commercial oleic acid methyl ester. No further efforts were made to identify structural differences of either fatty acid. We assumed that both were isomers of the fatty acid 18:1 (oleic acid). The two peaks for oleic acid could also be found in the GC-chromatograms for the underivatized orange juice extracts and were not artifacts of derivatization.

Ratios of experimental peak areas to internal standard (heptadecanoic acid methyl ester) were calculated and compared to an external calibration standard of all identified fatty acids for quantitative analysis. For oleic acid the sum of both isomers was determined and calculated with the response factor of commercially available oleic acid or oleic acid methyl ester.

Peel test (adhesion measurement)

The specimens were mounted in an Instron tensile testing machine (Instron Ltd., Stockholm, Sweden) with a 180° peel angle. The peel angle was kept at 180° during the test by using a supporting grip. The cross-head speed was 100 mm/min. The force was plotted vs the cross-head movement. The mean force for a stable plateau following the initial delamination peak was used for further calculations. Results were nor-
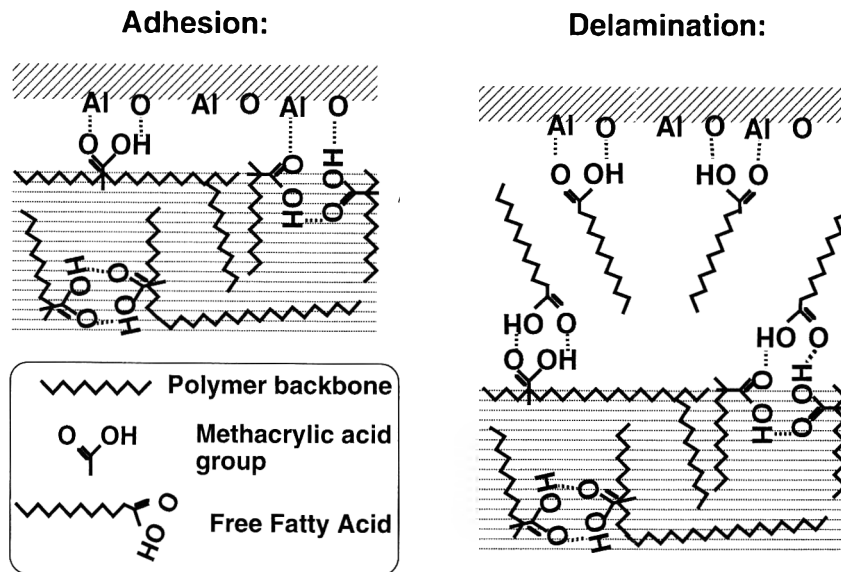
Adhesion:

Delamination:

Fig. 5—Delamination mechanism schematic.

malized to N/m by dividing by the strip width expressed in meters. The arithmetic mean value for the 5 strips was calculated for each sample.

RESULTS & DISCUSSION

FFAs WERE PRESENT IN ORANGE JUICE at <50 ppm when packed. This concentration in orange juice was not critical for adhesion in laminates of the type we used. During storage the level increased steadily with time and temperature to ≈200 ppm (Fig. 2). In parallel, the amount of FFAs absorbed in the packaging material increased with time and temperature (Fig. 3). As an apparent consequence of interaction with the FFAs, the adhesion between inside plastic layers and the alufoil decreased (Fig. 4). The peel values for each sample varied substantially as adhesion level started to drop. This reflected the fact that delamination started in a non-homogeneous way, with local initial delamination.

Upon reaching certain critical concentrations of FFAs in the packaging material, total delamination occurred. The critical concentration in the inside coating was 1.1–1.4% for all temperatures. The composition of the FFAs in orange juice and packaging material was essentially the same (Table 1) during storage at all temperatures. The saponified orange juice extracts (total fatty acids) were slightly different from the FFAs composition with respect to relative weight % of palmitic acid and linoleic acid.

The differences in the fatty acids concentration in weight % between orange juice extracts (FFAs) and saponified orange juice extracts (total fatty acids) decreased with time and increasing temperature. However, the absolute difference in mg/L between them stayed almost constant. This was an indication that the formation of FFAs was not due to a hydrolysis of these fatty acid esters. However, the polar lipid fraction of phospholipids had not been extensively extracted from the juices due to its relative insolubility in ether. This fraction has been shown to be the major source of the increase of FFAs during storage of orange juice (Nagy and Nordby, 1970). In general, the fatty acids composition agreed well with results reported for lipid composition of orange juice sacs (Kealey and Kinsella, 1979).

CONCLUSIONS

THE FORMATION AND ABSORPTION of FFAs correlated with loss of adhesion. The proposed mechanism for delamination suggested that the carboxylic groups of the FFAs interfered with

the acid/base bonds between aluminum foil and the tie resin (Fig. 5). The hydrophobic chain of the FFAs promoted the permeation into the polymer and the formation of a weak boundary layer between aluminum foil and the tie resin. The proposed mechanism corresponded to the acid/base interfacial interaction theory. When FFAs are present in food appropriate multilayer material structures must be selected to avoid delamination and assure acceptable quality over the shelf life. Structural changes in the nature, the co-monomer content or the amount of tie polymer could help to improve adhesion and resistance against delaminating. Multilayer flexible packages for orange juice were designed such that loss of adhesion is usually no problem under commercial storage at ambient temperatures.

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Ms received 1/30/95; revised 4/12/95; accepted 4/21/95.

Unsaturated Aldehydes Identification from Green Coffee

J. BOOSFELD and O.G. VITZTHUM

ABSTRACT

The dienals in green coffee beans were isolated by simultaneous distillation/extraction (SDE) and for artifact-free isolation by high vacuum distillation (HVD). Characterization and identification of compounds were by GC effluent sniffing, GC-MS, GC-FTIR and NMR spectroscopy. Isomers were determined either on-line from green coffee extract by direct deposition GC-FTIR or off-line after enrichment by automatic preparative GC and subsequent GC-FTIR and NMR spectroscopy. Two pairs of unsaturated aldehydes were identified: (E,E)-2,4- and (E,Z)-2,4-nonadienal as well as (E,E)-2,4- and (E,Z)-2,4-decadienal.

Key Words: green coffee, roasted, unsaturated, aldehydes, fatty acids

INTRODUCTION

THE VOLATILE AROMA of roasted coffee is mainly generated during roasting. Nevertheless, some volatile components are present in the green coffee. They contribute to the typical green coffee odor and may impact the cultivar-related flavor or off-flavor characteristics in corresponding roasted coffee (Becker et al., 1988; Holscher, 1991; Holscher and Steinhart, 1994; Liardon et al., 1990; Spadone et al., 1990; Vitzthum et al., 1976, 1990). Our objective was to elucidate certain unsaturated aldehydes of green coffee that are generated probably via autoxidation of the unsaturated fatty acids and esters (Grosch, 1987; Ullrich et al., 1988). The chemical identification and structural elucidation was by means of gas chromatographic (GC) separation and spectroscopic methods. Isolation of double bond isometric structures was by nondestructive high vacuum techniques.

MATERIAL & METHODS

Materials

Colombian green coffee was ground with a Condux® mill to a particle size 0.5-1 mm. Reference materials (E,E)-2,4-nonadienal and (E,E)-2,4-decadienal were supplied by Aldrich (Steinheim, Germany).

Sample preparation

Volatile compounds were isolated with an apparatus as described by Schieberle (1985) as well as by simultaneous distillation/extraction (SDE) for 2 hr according to Schultz et al. (1977). For isolation of the volatile complex for on-line GC-MS and GC-FTIR evaluations, ground green Colombian coffee (150 g) was placed inside a 2L round bottom flask. Distilled water (1 L) as well as internal standards 2,3-dimethoxytoluene and 2,3-dimethylpyrazine were added. SDE was carried out using a mixture of n-pentane/diethylether (1 + 1, v/v) as extracting agent. The raw extract was dried over anhydrous sodium sulfate and concentrated to about 0.5 mL by means of a Vigreux column (200 × 10 mm). This concentrate was prefractionated by means of column chromatography (column 250 × 10 mm; silica gel 60, particle size 0.063–0.200mm, Merck, Darmstadt, Germany). Elution was carried out first with 50 mL pentane (discarded). Then n-pentane/methylene chloride (1 + 4, v/v 50 mL) was used as eluting agent for fraction F2 and, subsequent, diethylether (50 mL) was used to obtain fraction F3. Fraction F2 was concentrated to about 0.5 mL and 1μL was injected into GC-MS, GC-FTIR and GC-olfactometry.

The apparatus for artifact-free aroma isolation via HVD consisted of a 2L round bottom flask and two cold traps cooled with liquid nitrogen.

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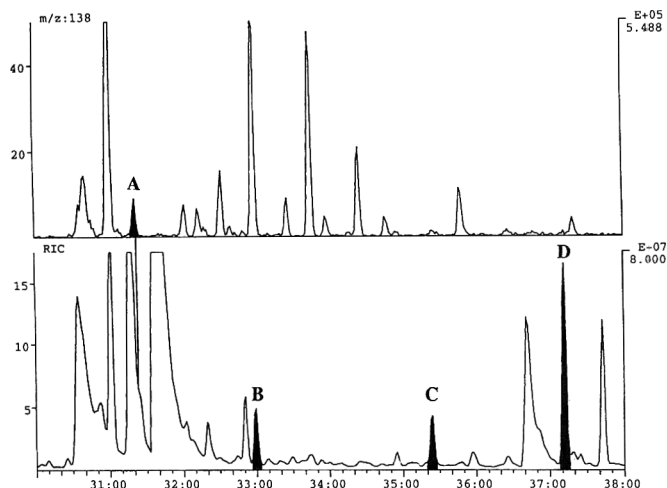
Fig. 1—Mass spectral chromatogram of SDE extract of Colombian green coffee; total ion chromatogram (below) and chromatogram of m/z = 138 (above).

Colombian ground green coffee (600 g) was placed in a 2L round bottom flask. n-pentane/diethylether (1 + 1, v/v 50 mL) was added to the green coffee and carefully dispersed. The flask was frozen with liquid nitrogen and connected to the vacuum distillation apparatus. Vacuum distillation was carried out for 2 hr at room temperature and thereafter 5 hr at 75°C at a final inner pressure of 32 Pa. After melting the condensate was collected in a separatory funnel. The condensed water was drawn off and extracted three times with diethylether. The combined organic layers were dried over anhydrous sodium sulfate and concentrated as described. This concentrate was prefractionated into two fractions by column chromatography on silica gel. Elution was carried out first with 50 mLn-pentane (fraction 1, discarded) and then with 50 mL diethylether (fraction 2). Fraction 2 was concentrated to a volume of about 100 μL for GC-MS, GC-FTIR and GC-olfactometry.

Gas chromatography

Gas chromatographic separations were performed on HP 5890 II gas chromatographs equipped with a DB-Wax capillary column (60 m × 0.25 mm; 0.25 μm film thickness). Helium was carrier gas at 1-2 mL/min; temperature program: 35°C for 2 min, then 40°C/min to 60°C, then 3°C/min to 215°C, hold for 15 min. A sample volume of 3 μL was injected via a temperature programmable injection system (60°C to 200°C; 12°C/sec) and GC sniffing was performed by splitting the GC effluent (in a ratio 1:1) to a flame ionization detector and a sniffing port.

Mass spectrometry, FTIR spectroscopy

Mass spectroscopy was performed on a Finnigan MAT 95 Q; mass spectra were generated at 70eV in the electron impact mode. Fourier Transform Infra Red (FTIR) spectra were recorded by a BIO-RAD Tracer interface using the direct deposition technique in combination with the 'FTS45' FTIR spectrometer. The transfer line was held at 250°C and the slide was cooled down to -196°C with liquid nitrogen (Bourne et al., 1990; Diederich et al., 1991; Haefner et al., 1988).

Isolation of (E,Z)-2,4-nonadienal and (E,Z)-2,4-decadienal

(E,Z)-2,4-Nonadienal as well as (E,Z)-2,4-decadienal may occur as impurities of their corresponding synthetic (E,E)-2,4-isomers (Aldrich, Steinheim, Germany). Isolation of such compounds was performed by
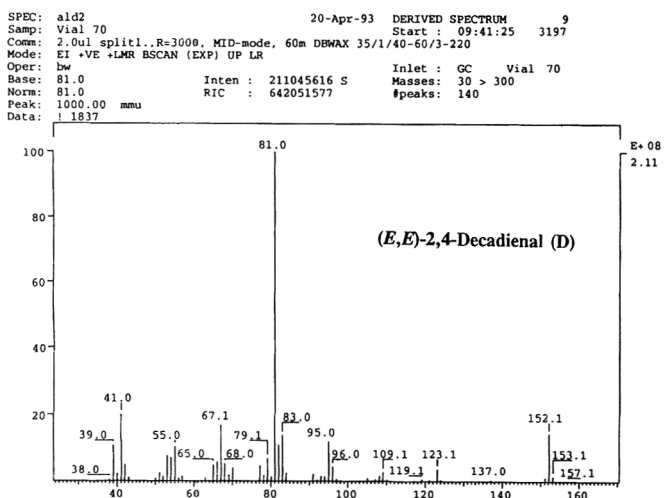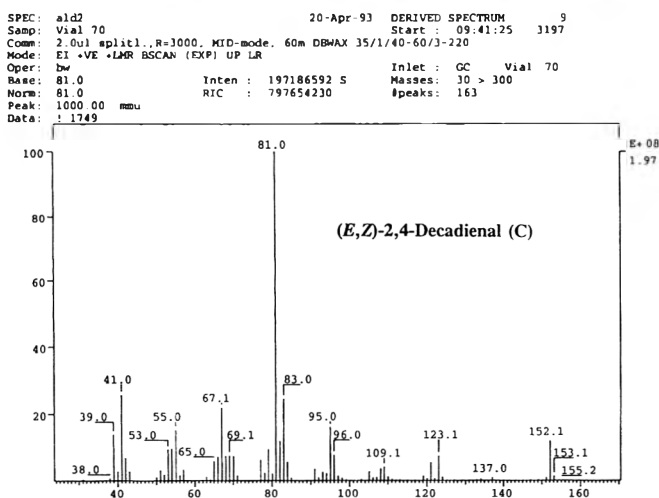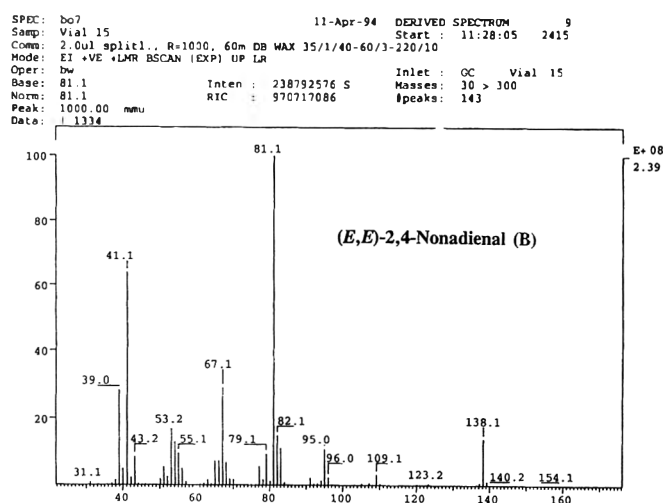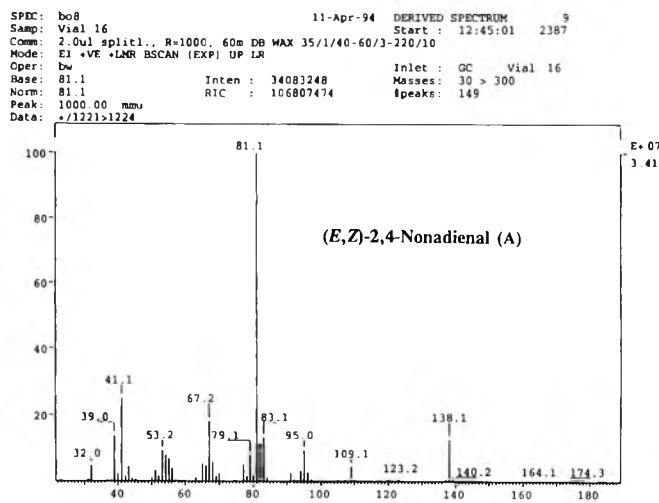
SPEC: bo8   11-Apr-94 DERIVED SPECTRUM   9
Samp: Vial 16   Start : 12:45:01   2387
Comm: 2.0ul splitl., R=1000, 60m DB WAX 35/1/40-60/3-220/10
Mode: EI +VE +LMR BSCAN (EXP) UP LR
Oper: bw   Inlet : GC   Vial 16
Base: 81.1   Inten : 14083248   Masses: 30 > 300
Norm: 81.1   RIC   : 106807474   #peaks: 149
Peak: 1000.00 mmu
Data: +/1221>1224

(E,Z)-2,4-Nonadienal (A)

SPEC: bo7   11-Apr-94 DERIVED SPECTRUM   9
Samp: Vial 15   Start : 11:28:05   2415
Comm: 2.0ul splitl., R=1000, 60m DB WAX 35/1/40-60/3-220/10
Mode: EI +VE +LMR BSCAN (EXP) UP LR
Oper: bw   Inlet : GC   Vial 15
Base: 81.1   Inten : 238792576 S   Masses: 30 > 300
Norm: 81.1   RIC   : 970717086   #peaks: 143
Peak: 1000.00 mmu
Data: I 1334

(E,E)-2,4-Nonadienal (B)

SPEC: ald2   20-Apr-93 DERIVED SPECTRUM   9
Samp: Vial 70   Start : 09:41:25   3197
Comm: 2.0ul splitl.,R=3000, MID-mode. 60m DBWAX 35/1/40-60/3-220
Mode: EI +VE +LMR BSCAN (EXP) UP LR
Oper: bw   Inlet : GC   Vial 70
Base: 81.0   Inten : 197186592 S   Masses: 30 > 300
Norm: 81.0   RIC   : 797654230   #peaks: 163
Peak: 1000.00 mmu
Data: ! 1749

(E,Z)-2,4-Decadienal (C)

SPEC: ald2   20-Apr-93 DERIVED SPECTRUM   9
Samp: Vial 70   Start : 09:41:25   3197
Comm: 2.0ul splitl.,R=3000, MID-mode, 60m DBWAX 35/1/40-60/3-220
Mode: EI +VE +LMR BSCAN (EXP) UP LR
Oper: bw   Inlet : GC   Vial 70
Base: 81.0   Inten : 211045616 S   Masses: 30 > 300
Norm: 81.0   RIC   : 642051577   #peaks: 140
Peak: 1000.00 mmu
Data: ! 1837

(E,E)-2,4-Decadienal (D)

Fig. 2—MS spectra of 2,4-nonadienals and 2,4-decadienals.

GC effluent cryo-trapping using a preparative *Gerstel* 'Multi Column Switching' system (Gerstel, Mühlheim, Germany). The isolate was eluted with CDCl₃ and GC-FTIR and NMR spectroscopy was carried out using the BIO-RAD *Tracer* interface and a *Bruker* AC-250 MHz spectrometer, respectively. NMR conditions were 512 scans for (E,Z)-2,4-decadienal with an acquisition time of $\approx$ 17 min and 117.500 scans for (E,Z)-2,4-nonadienal with an acquisition time of $\approx$ 69 hr.

RESULTS & DISCUSSION

THE VOLATILE AROMA COMPLEX of roasted coffee has been broadly investigated. This contrasts with the relatively few published reports on the volatile fractions of green coffee. This is a consequence of the fact that coffee is used after roasting. Vitzthum et al. (1976) related the typical green coffee odor to 2-methoxy-3-*iso*propyl- and 2-methoxy-3-*iso*butyl pyrazine. A variety of saturated and unsaturated aldehydes were identified by Guyot et al. (1983) and Spadone and Liardon (1988). The latter compounds are known decomposition products of the autoxidation of fatty acids. Their occurrence in green coffee was expected since the total lipid content in green coffee amounts to about 15% in Arabica and 10% in Robusta and about 1/3 to 1/2 the fatty acid complex is formed by linoleic acid (Maier, 1981).

In the mass spectral chromatogram pairs of GC peaks of a green coffee aroma extract were of special interest (peaks A-D). In the total ion chromatogram (Fig. 1) peak A appeared only as a shoulder of the adjacent peak but it gave a sole peak in the MS chromatogram of m/z = 138. The sensory impressions of these products A-D at the sniffing port were distinctively different and varied from *metallic, fried, flowery* to *oily*. Mass spec-

Table 1—Relative amounts of (E,E)-2,4-nonadienal, (E,Z)-2,4-nonadienal, (E,E)-2,4-decadienal and (E,Z)-2,4-decadienal in HVD and SDE extracts of Colombian green coffee

Peak	Compound	m/e	HVD (% of total peak area)	SDE (% of total peak area)
A	(E,Z)-2,4-nonadienal	138	0.40	0.12
B	(E,E)-2,4-nonadienal	81	48.68	9.89
C	(E,Z)-2,4-decadienal	81	5.19	5.48
D	(E,E)-2,4-decadienal	81	16.82	38.38

tra of the corresponding pairs suggest 2,4-nonadienal and 2,4-decadienal isomers, respectively. However, their fragmentation patterns were similar to a large extent and indicated the presence of double bond isomeric structures of both pairs of 2,4-dienals. (E,E)-2,4-nonadienal (B) as well as (E,E)-2,4-decadienal (D) have been identified in green and roasted coffee (Holscher et al., 1990; Holscher, 1991). The chemical structure of the isomer (E,Z)-2,4-decadienal (C) has been identified from green coffee aroma extracts (Boosfeld et al., 1993). By this our results, its occurrence in green coffee was proven by isolation with a non-destructive high vacuum distillation procedure. The mass spectra of the two pairs of peaks (Fig. 2a–d) show peak A and B as well as C and D, appeared to be identical to a large extent and showed only little differences in intensities of certain mass peaks. Comparison of the sensory impressions, the mass spectral and retention data of peak B and D with published data and reference substances confirmed the chemical structure of peak B as (E,E)-2,4-nonadienal and of peak D as (E,E)-2,4-decadienal.

This similarity of mass spectra of the clearly separated peaks A and B as well as of the peaks C and D, respectively, was
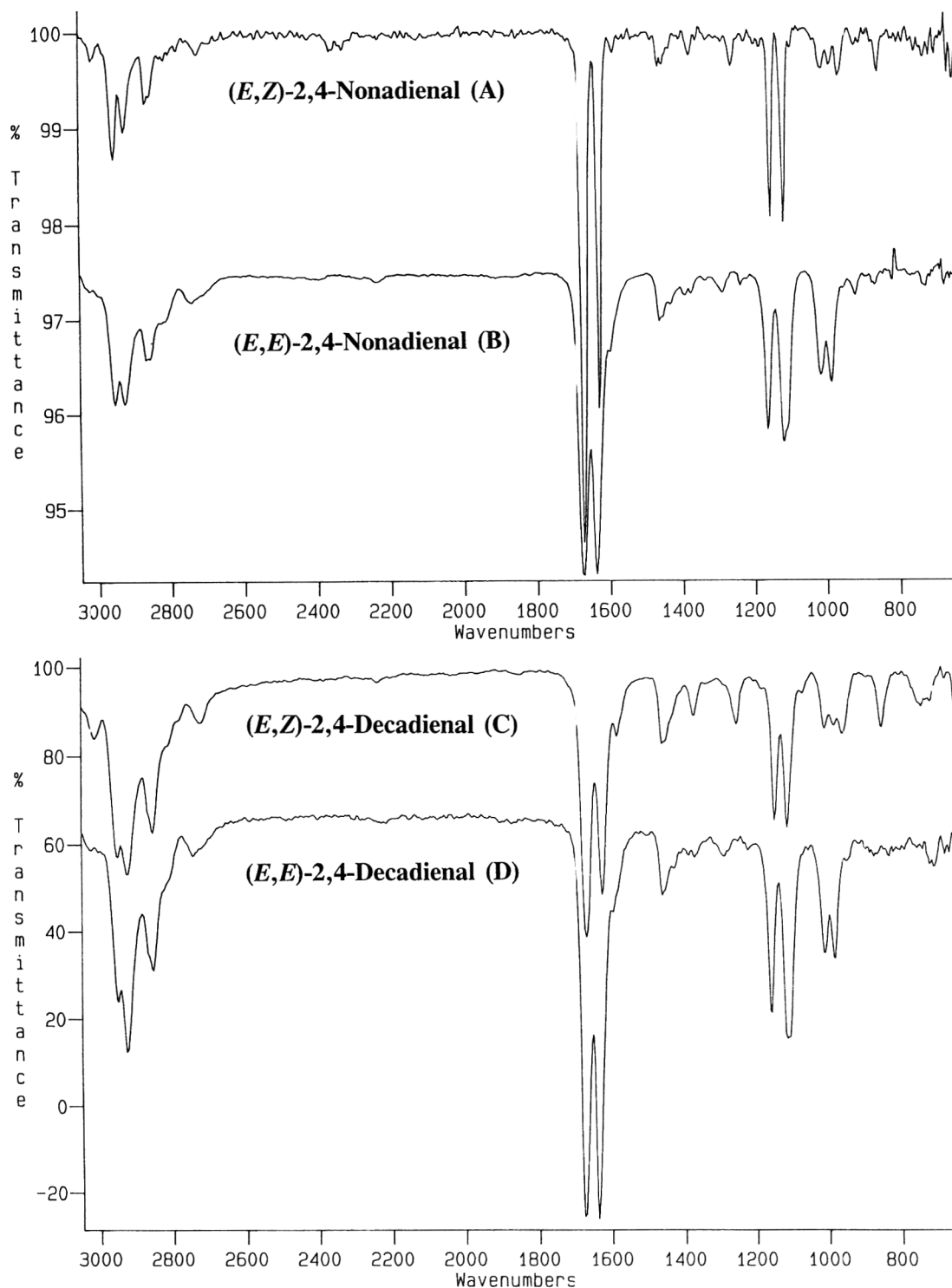
Fig. 3—Tracer-FTIR solid phase spectra of 2,4-nonadienals and 2,4-decadienals.

clear evidence for the different orientations of one of the double bonds in the carbon chain of the dienals (E/Z isomeric structures). The conditions in the mass spectrometer led to isomerization of alkenyl double bonds. As a result mass spectroscopy could not distinguish between E/Z isomers. Fundamentally, we could depict four E,Z isomers of each 2,4-dienal structure-E,E, E,Z, Z,E and Z,Z. However, the comparison of retention data of peak **C** with published data (Pfannhauser, 1990) revealed the presence of (E,Z)-2,4-decadienal which was confirmed by on-line GC-FTIR and NMR spectroscopy (Boosfeld et al., 1993). The presence of (E,Z)-2,4-nonadienal (**A**) in green coffee aroma extracts has not been reported. In this work, the isolation and structural identification of γ,δ-(Z) 2,4-nonadienal was by means of SDE and HVD and after preparative enrichment the identi-

fication of its chemical structure was by GC-FTIR and NMR spectroscopy. The relative amounts of the two 2,4-nonadienals (**A** and **B**) as well as of the two 2,4-decadienals (**C** and **D**) were distinguished by MS as percentage areas relative to total peak areas (Table 1).

The wavenumbers of absorption bands of the solid phase Tracer FTIR spectra of peaks **A-D** (Fig. 3a–b) and some selected assignments to the type vibration of the two pairs of isomeric 2,4-nonadienals and 2,4-decadienals were determined (Table 2 and 3). As expected, the FTIR spectra of the structural related pairs of dienals-(E,E)-2,4-nonadienal and (E,E)-2,4-decadienal as well as of (E,Z)-2,4-nonadienal and (E,Z)-2,4-decadienal—were similar to a large extent in the range from wave number 3500 to 1300. In general, isomeric double bonds

Table 2—*Tracer*-FTIR spectral data of (E,E)-2,4-nonadienal and (E,Z)-2,4-nonadienal

| | (E,E)-2,4-nonadienal | | | (E,Z)-2,4-nonadienal | |
[cm⁻¹]	Intensity[a]	Assignment	[cm⁻¹]	Intensity[a]	Assignment
3342.3	w	2ν C=O	3325.5	w	2ν C=O
3050-2700	s	ν C–H	3050-2700	s	ν C–H
2746.2	w	ν OC–H	2728.3	w	ν OC–H
1675.8	vs	ν C=O / C=C	1674.0	vs	ν C=O / C=C
1640.0	vs	ν C=O / C=C	1631.0	vs	ν C=O / C=C
1602.7	m	ν C=O / C=C	1590.5	m	ν C=O / C=C
1465.0	m	$\delta_{as, s}$ CH₃, CH₂	1464.3	m	$\delta_{as, s}$ CH₃, CH₂
			1380.5	m	
			1264.4	m	
1167.6	s		1157.7	s	
1124.4	s		1122.0	s	
1021.2	s		1019.2	m	
992.9	s	$\delta_{o.o.p.}$ (E) C=C	994.0	m	$\delta_{o.o.p.}$ (E) C=C
			860.9	m	$\delta_{o.o.p.}$ (Z) C=C
			757.5	m	
			652.8	m	

[a] Intensities: w = weak, m = medium, s = strong, vs = very strong.

Table 3—*Tracer*-FTIR spectral data of (E,E)-2,4-decadienal and (E,Z)-2,4-decadienal

| | (E,E)-2,4-decadienal | | | (E,Z)-2,4-decadienal | |
[cm⁻¹]	Intensity[a]	Assignment	[cm⁻¹]	Intensity[a]	Assignment
3339.9	w	2ν C=O	3334.7	w	2ν C=O
3050-2700	s	ν C–H	3050-2700	s	ν C–H
2745.9	w	ν OC–H	2725.6	w	ν OC–H
1676.5	vs	ν C=O / C=C	1674.0	vs	ν C=O / C=C
1639.6	vs	ν C=O / C=C	1630.7	vs	ν C=O / C=C
1602.2	m	ν C=O / C=C	1589.3	w	ν C=O / C=C
1465.1	m	$\delta_{as, s}$ CH₃, CH₂	1464.7	m	$\delta_{as, s}$ CH₃, CH₂
			1379.0	m	
			1260.9	m	
1166.7	s		1156.9	s	
1121.6	s		1122.3	s	
1020.2	s		1018.7	m	
993.1	s	$\delta_{o.o.p.}$ (E) C=C	993.5	m	$\delta_{o.o.p.}$ (E) C=C
			862.1	m	$\delta_{o.o.p.}$ (Z) C=C
			752.7	m	
			653.4	m	

[a] Intensities: w = weak, m = medium, s = strong, vs = very strong.

Table 4—NMR spectral data of (E,E)-2,4-nonadienal and (E,Z)-2,4-nonadienal[a]

| | | (E,E)-2,4-nonadienal | | | | (E,Z)-2,4-nonadienal | |
δ [ppm]	Mult.	Assignm.	^3J [Hz]	δ [ppm]	Mult.	Assignm.	^3J [Hz]
9.49	d	H¹ (CHO)	H¹H² = 8.3	9.59	d	H¹ (CHO)	H¹H² = 7.9
6.04	dd	H²	H²H³ = 15.4	6.13	dd	H²	H²H³ = 15.1
7.05	m	H³		7.43	ddd	H³	H³H⁴ = 11.7
6.23-6.29	m	H⁴, H⁵		6.25	dt	H⁴	H⁴H⁵ = 10.8
2.19	qa	H⁶	H⁶H⁷ = 7.1	6.00	dt	H⁵	H⁵H⁶ = 7.8
1.24-1.47	m	H⁷, H⁸		2.33	dq	H⁶	H⁶H⁷ = 7.8
0.88	t	H⁹	H⁸H⁹ = 7.1	1.35	m	H⁷, H⁸	
				0.91	t	H⁹	H⁸H⁹ = 7.1

[a] Chemical shifts [ppm, CDCl₃], assignments and coupling constants [$^3J_{HH}$] (d = doublet, dd = double doublet, t = triplet, qa = quartet, qi = quintet, m = multiplet)

Table 5—NMR spectral data of (E,E)-2,4-decadienal and (E,Z)-2,4-decadienal[a]

| | | (E,E)-2,4-decadienal | | | | (E,Z)-2,4-decadienal | |
δ [ppm]	Mult.	Assignm.	^3J [Hz]	δ [ppm]	Mult.	Assignm.	^3J [Hz]
9.49	d	H¹ (CHO)	H¹H² = 7.8	9.59	d	H¹ (CHO)	H¹H² = 7.8
6.03	dd	H²	H²H³ = 15.2	6.13	dd	H²	H²H³ = 15.2
7.04	dd	H³	H³H⁴ = 10.0	7.42	dd	H³	H³H⁴ = 11.2
6.22-6.29	m	H⁴, H⁵		6.25	t (br.)	H⁴	H⁴H⁵ = 11.0
2.17	qa	H⁶	H⁵H⁶ = 6.4	5.99	dt	H⁵	H⁵H⁶ = 7.8
1.42	qi	H⁷	H⁶H⁷ = 7.0	2.32	qa	H⁶	H⁶H⁷ = 6.6
1.18-1.35	m	H⁸, H⁹		1.0-1.6	m	H⁷, H⁸, H⁹	
0.85	t	H¹⁰	H⁹H¹⁰ = 6.8	0.89	t	H¹⁰	H⁹H¹⁰ = 6.8

[a] Chemical shifts [ppm, CDCl₃], assignments and coupling constants [$^3J_{HH}$] (d = duplet, dd = double duplet, t = triplet, qa = quartet, qi = quintet, m = multiplet; br. = broad).

scarcely affect vibrations caused by the aliphatic part of the molecule and strength of bonds. The most important spectral differences between (E,E) and (E,Z) structures were obvious in the fingerprint region below 1100 wave numbers. After normalizing the region of the CH vibrations in the range 3050 to 2700 cm⁻¹ it was obvious that the $\delta_{o.o.p.}$ absorption band for the (E) double bond existed for both pairs of dienals at wave numbers 992 to 994. These bands have roughly half the intensity for the (E,Z)-dienals as in the spectra of the (E,E)-dienals. Furthermore, the $\delta_{o.o.p.}$ absorption band for the (Z) double bond

(860.9 and 862.1 cm^{-1}) occurred only in the spectra of compounds A and C. We could assume that the structure of these dienal isomers were (E,Z)- or (Z,E)-2,4-nonadienal and (E,Z)- or (Z,E)-2,4-decadienal, respectively.

Preparative enrichment of (E,Z)-2,4-nonadienal and (E,Z)-2,4-decadienal from authentic reference samples

The final confirmation of a chemical structure requires the availability of reference material. Generally, this becomes a problems if authentic reference substances are difficult to obtain in a pure state. We found that the commercially available synthetic (E,E)-2,4-nonadienal as well as the (E,E)-2,4-decadienal contained substantial amounts of isomeric compounds with similar retention data and sensory properties as substances A and C. Therefore, these impurities were isolated by automatic GC effluent cryo-trapping in the lower nanogram range for subsequent off-line GC-FTIR spectroscopy (in the case of E,Z-2,4-nonadienal) and in the mg range for NMR spectroscopic evaluations.

The final structural confirmation of isolates was performed via NMR spectroscopy and the compounds were identified as (E,Z)-2,4-decadienal and (E,Z)-2,4-nonadienal, respectively. For determination between E/Z isomers the most important basis is the $^3J_{HH}$ coupling constant (see Table 4 and 5). It was obvious that the protons H^{2}H^3 in the NMR spectrum of (E,Z)-2,4-decadienal and (E,Z)-2,4-nonadienal, respectively, were located in an (E) and H^{4}H^5 in a (Z) configuration because of the bigger coupling constants $^3J_{(H^2H^3)}$ compared to $^3J_{(H^4H^5)}$. As a result of the smaller coupling constant $^3J_{(H^4H^5)}$ the multiplicity of signals for H^4 for both (E,Z)-dienals were similar to a triplet because of the similar values of $^3J_{(H^3H^4)}$ and $^3J_{(H^4H^5)}$. In comparison to that we found a double doublet for the protons H^2 and H^3 because of the different values of $^3J_{(H^2H^3)}$ and $^3J_{(H^3H^4)}$. The overall appearance of the NMR spectrum of (E,E)-2,4-nonadienal and (E,Z)-2,4-nonadienal as well as (E,E)-2,4-decadienal and (E,Z)-decadienal demonstrated the differences between the two isomers.

The assignment of NMR signals to corresponding protons of the two isomers of (E,E)- and (E,Z)-2,4-decadienal was achieved using the ^{1}H, ^{1}H-COSY NMR spectra.

Only the combination of several spectroscopic and chromatographic methods and techniques provided clear evidence for the orientation in the double bond isomeric structures. With authentic reference material, the peaks B and D were clearly identified as (E,Z)-2,4-nonadienal and (E,Z)-2,4-decadienal, respectively, via comparison with sensory properties, retention and spectroscopic data.

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Ms received 1/31/95; revised 3/31/95; accepted 4/21/95.

Highly Sensitive Method for Urea Detection in Wine

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——————— ABSTRACT ———————

Trace amounts of urea (less than 50 µg/L) in wine were accurately determined using a modified high performance liquid chromatography (HPLC-fluorometric) technique. Appropriate selection of eluent, HPLC columns, and chiral derivatives for the o-phthalaldehyde solution provided a 20-fold enhancement of sensitivity over established methods. A linear relationship was observed at concentrations from 0.5–500 µg/L of aqueous urea solution. The threshold level for detectability of urea in wine was 5 µg/L. The method is simple enough to be applicable to most beverages.

Key Words: wine, urea, HPLC, fluorometric, beverages

INTRODUCTION

IN 1986, the Canadian Ministry of Health and Welfare issued regulations limiting the amount of ethyl carbamate (EC), a well known carcinogen (Mirvish, 1968; Inai et al., 1991), in alcoholic beverages. In the U.S.A., very low target acceptable levels of EC have been agreed upon by domestic wine producers. Since urea is an essential precursor of EC, lowering EC concentration in the finished products depends on reducing the concentration of urea during manufacturing accomplished by enzymatic conversion (Ough and Trioli, 1988; Fujinawa et al., 1990, 1992a; Kodama et al., 1991, 1994).

Acid urease derived from *Lactobacillus fermentum* works at pH 3.0 to 4.0 and can efficiently lower urea content in beverages (Kakimoto et al., 1990). Acid urease has been approved by the Japanese Tax Administration Agency (1987) for use in all alcoholic beverages and has been categorized as Generally Recognized as Safe (GRAS) for use in wine, including sake, by the Food and Drug Administration of the USA (1992).

A highly sensitive method is required to detect residual urea in wine after enzymatic removal. Commonly used enzymatic and colorimetric methods have detection limits of ≈ 1.0 mg/L (Almy and Ough, 1989; Nagel and Weller, 1989). Such methods are not sensitive enough to be useful for trace residual urea (< 1 mg/L) in wine.

We previously described a urea determination method with a detection limit of 50 µg/L in wine (Fujinawa et al., 1992b). The method was both time and labor intensive because solutions had to be concentrated until the urea content reached detectable levels. Our current objective was to develop a method for rapid and highly sensitive determination of urea content of aqueous solutions, and determine its usefulness for measurement of trace urea in wine.

MATERIALS & METHODS

Reagents

All reagents were of reagent grade and were used without further purification. Distilled water for HPLC was obtained from Wako Pure Chemical Industries Ltd. (Osaka, Japan).

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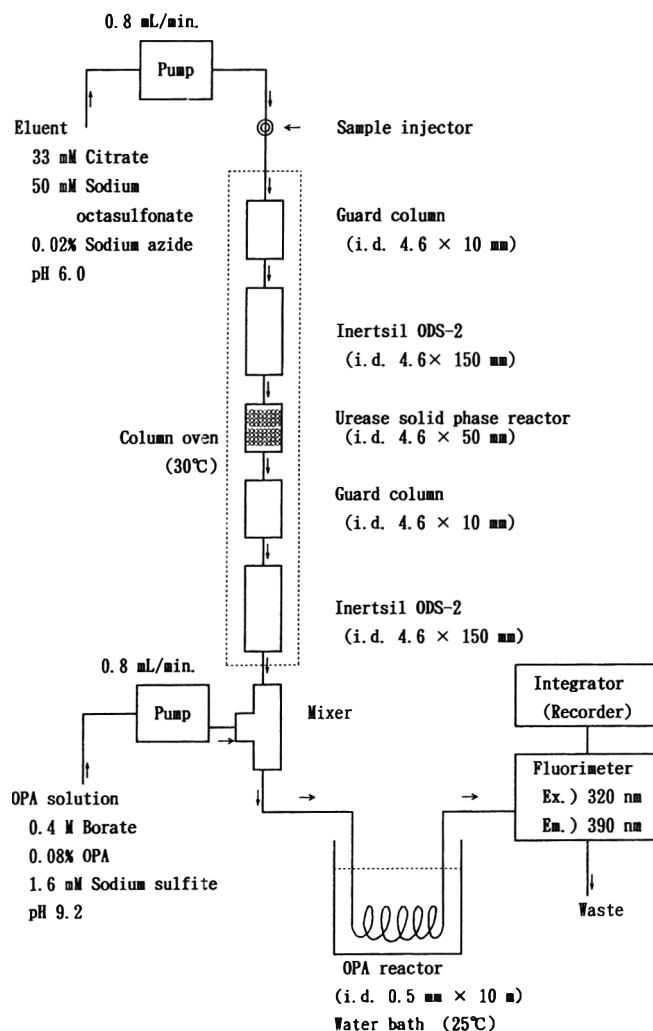
Fig. 1—Schematic of the HPLC-fluorometer system. Details in Materials & Methods.

Wines and determination of initial urea content

Japanese red and white table wines, and an imported California sherry, all commercially bottled, were purchased from retailers in Japan. Initial urea contents were determined by the urea/ammonia UV method using an enzymatic test kit (Boehringer Mannheim GmbH, Mannheim, Germany).

Trace urea determination

Wine samples (10 mL) were loaded onto a 4 mL column (5 × 100 mm, BIO-RAD, Calif.) of Amberlite CG120 Type 1 (H+ form, Rohm & Haas Co., Philadelphia, PA). The column was washed with 14 mL of distilled water, with 3 mL of 33 mM citrate buffer (pH 6.0), and then eluted with 28 mL of 33mM citrate buffer (pH 6.0). After addition of 1 mL of 2.7N KOH containing 0.6% (w/v) sodium azide, the eluate was brought to 30 mL with distilled water. Test solution (20 µL) was injected into an HPLC-fluorometer system (Fig. 1).

The HPLC-fluorometer system was similar to that previously reported. Improvements include (1) a Hitachi L-6000 HPLC system and a Hitachi
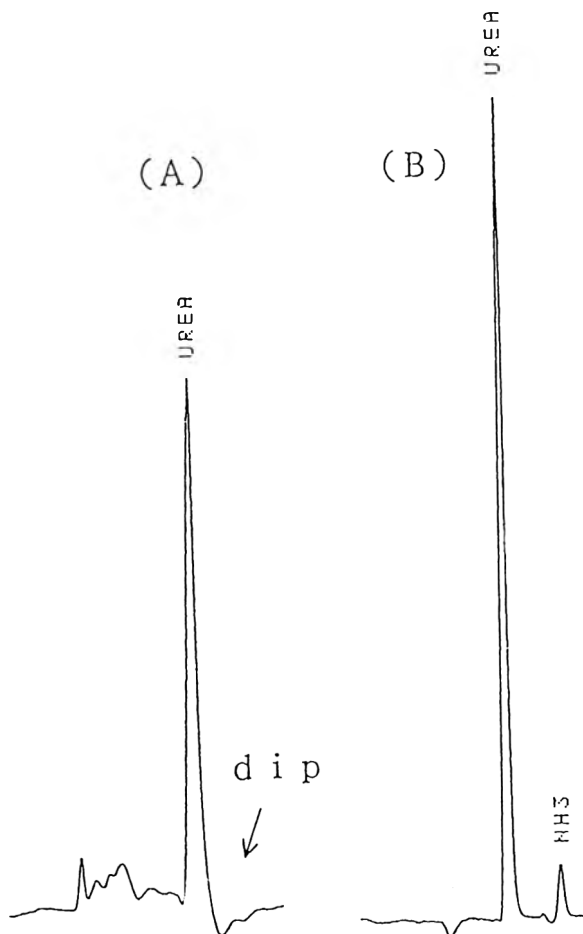
Fig. 2—Chromatograms of non-neutralized (A) and neutralized (B) eluate. Each was prepared from 250 μg/L of urea solution.

Table 1—Urea peak area detected in neutralized eluates[a] prepared from Japanese red wine

Added sodium azide (%)	Peak area[b]	
	Initial	After 7 days at 25°C
0	236,963	0
0.02	223,591	222,629

[a] Neutralized eluates described in Materials & Methods.
[b] Relative responses from an integrator (Hitachi D-2500).

F-1080 Fluorescence Detector were used (Hitachi, Tokyo, Japan). Two pieces of Inertsil ODS-2 (5 μm, 4.6 × 150 mm, GL Science Inc., Tokyo, Japan) were used to separate ammonia from urea or substances which gave fluorescence, and two guard columns with the resin were employed to protect the HPLC system. (2) A citrate buffer (33 mM citrate, pH 6.0, 50 mM sodium octasulfonate, 0.02% (w/v) sodium azide) was used as eluent instead of the phosphate buffer. (3) The o-phthalaldehyde (OPA) solution containing N-acetylcysteine was replaced with a borate buffer (400 mM borate, pH 9.2, 0.08% (w/v) OPA, 1.6 mM sodium sulfite). (4) The excitation wavelength was 320 nm, and the observed emission wavelength was 390 nm.

Removal of urea by acid urease in wine and recovery test

Acid urease derived from *Lactobacillus fermentum* (3.5 units/mg, Takeda Chemical Industries, Ltd., Osaka, Japan) was used for enzymatic removal of urea. One unit of enzyme was defined as the enzymatic activity that liberated 1 μmole of ammonia from urea (5000 mg/L)/min in 100 mM citrate, pH 4.0 at 37°C. We added 1000 mg/L of the enzyme to wines (1440 mL) and incubated them for 72 hr at 25°C. After incubation, the enzymes were eliminated by filtration through nitro-cellulose (pore size: 0.2 μm, Toyo Roshi Kaisha, Ltd., Tokyo, Japan) prior to determination of urea content. The added urea in the test wines resulted in concentrations of 3, 5, 10, 50, 100, 250, and 500 μg/L.

RESULTS & DISCUSSION

Improvement of trace urea detection

In order to establish a more sensitive method of trace urea determination, we examined different combinations of eluents, types of columns in the HPLC system, and chiral derivatization agents in the OPA solution.

Eluents

Noisy background signals previously interfered with detection of weak urea-derived ammonia peaks at concentrations <50 μg/ L. We determined that the phosphate buffer used in previous studies (50 mM, pH 6.9) fluoresced enough to increase the baseline of the chromatogram above acceptable levels. A nonfluorescing citrate buffer (33 mM, pH 6.0) was therefore used. In addition, pH of the eluents was adjusted to 6.0, which optimized the activity of the immobilized urease (137 units/mg from Jack bean, Wako Pure Chemical Industries, Ltd., Osaka, Japan) to silica in a solid phase reactor. Under these conditions, urea was completely converted to ammonia and carbon dioxide.

In the pretreatment phase, the eluate became more acidic after passing through the cation-exchange resin column. This change in pH caused fluctuation in the baseline, with a dip just after the urea peak [Fig. 2 (A)]. The dip affected peak area determinations, particularly at low concentrations. The eluate was therefore neutralized by addition of 1 mL of 2.7N KOH. This eliminated the baseline dip [Fig. 2 (B)].

The urea content of neutralized eluate had decreased on standing a few days at room temperature (≈ 25°C). One reason for this may have been the activity of microorganisms which consumed the ammonia as nitrogen source, since no decrease was noted in an eluate allowed to remain acidic. The eluate was therefore kept sterile by addition of sodium azide dissolved in 2.7 N KOH at 0.6% (w/v). Additions of sodium azide to neutralized eluate prevented urea content from decreasing over time (Table 1).

Types of HPLC columns

A variety of reverse phase HPLC columns are commercially available. Several types were tested for ability to separate ammonia and urea. Residual silanol groups resulted in broadening or the tailing of the urea peak. The Inertsil ODS-2 column was therefore selected as optimal, because its elaborate endcapping minimized the effects of residual silanol groups after reverse phase chromatography, giving better peak resolution.

Chiral derivatization agents in OPA solution

OPA has been widely used in fluorometric analysis of amino acids and biologically active amines. OPA reacts with primary amines under alkaline conditions in the presence of thiol compounds, such as 2-mercaptoethanol, resulting in fluorescent compounds. Yasui and Hayashi (1990) reported that sodium sulfite gave derivatives of higher fluorescence than 2-mercaptoethanol in the reaction of OPA with ammonia. They observed an excitation wavelength of 320 nm for such derivatives, and an emission wavelength of 390 nm. In our previous method, N-acetylcysteine was used to form fluorescent derivatives. Chromatograms obtained for a urea solution (100 μg/L) reacted with an OPA solution containing N-acetylcysteine were compared with those containing sodium sulfite (Fig. 3). The OPA solution containing sodium sulfite enhanced the peak area of ammonia six times over that containing N-acetylcysteine. The addition of the compound gave derivatives of greater fluorescence than 2-mercaptoethanol. The use of sodium sulfite therefore increased the sensitivity by a factor of six.

In the previous method (Fujinawa et al., 1992b), eluates obtained after passing through a cation-exchange resin column were concentrated and adjusted to the initial volume (10 mL)
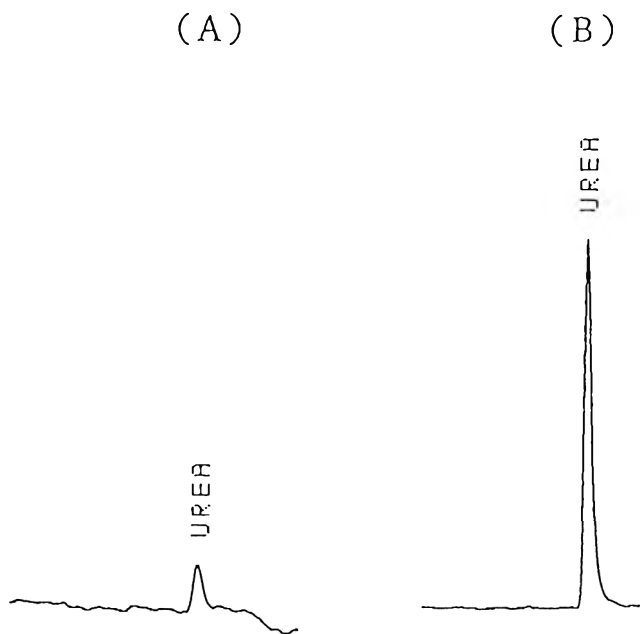
(A)   (B)

Fig. 3—Chromatograms of 100 µg/L urea solution reacted with an OPA solution containing N-acetylcysteine (A) or sodium sulfite (B). Peak area: (A) 638, 873; Peak area: (B) 3978, 333.
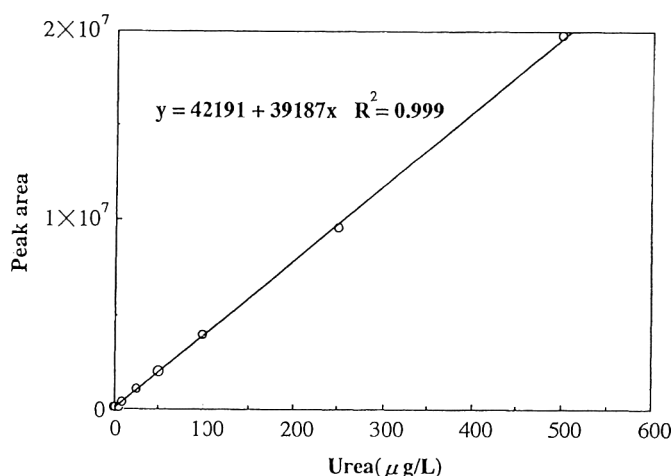

$$y = 42191 + 39187x \quad R^2 = 0.999$$

Fig. 4—Calibration curve of urea in distilled water. Coefficients of variation represent 1-5% in 0-500 µg/L of urea concentrations.

prior to quantification. In the improved method, we omitted the concentration operation and used the Hitachi F-1080 Fluorescence detector (Hitachi, Ltd. Tokyo, Japan) to monitor urea. This detector provides about a 10-fold increase in sensitivity over the older Shimadzu RF-520. As a result, urea determination method had almost a 20-fold increase in sensitivity.

Calibration curve for urea in distilled water by improved method

Urea dissolved in eluent at 0 (blank), 0.5, 1, 5, 50, 100, 250, and 500 µg/L, as standard solutions was injected into the HPLC fluorometer, without the pretreatment operation, as described above. Test experiments were performed in triplicate. The calibration curve of the arithmetic mean calculated from three independent experiments was plotted (Fig. 4). A linear response was noted at urea concentrations of 0–500 µg/L. Coefficients of variation of peak areas among three test experiments represented 5.5, 3.9, 3.3, 3.8, 1.1, 1.9, 3.6, 2.1, and 3.8% at respective concentrations of standard solutions from 0 to 500 µg/L.

Recovery test of urea added to sherry

Test sherries had urea added (3–500 µg/L) using 1 mL of urea solutions of varying concentration. The samples were subjected to pretreatments and then to HPLC-fluorometric analysis. Recovery tests were repeated three times at one test/day. Urea contents of test sherries were < 50 µg/L after decomposition by acid urease for 72 hr at 25°C. The ≈ 3 mg/L of naturally occurring urea in sherries was reduced to 42 µg/L by urea degradation using acid urease (Table 2). Recovery rates of urea from the experimental sherry samples (5–500 µg/L) ranged from 95 to 110%.

Recovery test of urea added to red and white wines

The naturally occurring urea in both red and white table wines was about 2 mg/L. Recovery tests for the red and white wines were carried out using the same procedure used for sherry. Addition of urease decreased the initial content of red wines to 39 µg/L, and of the white wine to 24 µg/L (Table 3 and 4). Recovery rates from samples with urea (5–500 µg/L) ranged from

Table 2—Recovery of urea from sherry

Urea added (µg/L)	Urea found[a] (µg/L)	Recovery[a] rate (%)	Coefficient of variation (%)
0 (blank)	42	—	5.0
3	44	67	0.9
5	47	100	1.0
10	53	110	5.6
50	90	96	3.2
100	137	95	5.6
250	282	96	5.8
500	543	100	3.8

[a] Mean of three independent tests.

Table 3—Recovery of urea from red wine

Urea added (µg/L)	Urea found[a] (µg/L)	Recovery[a] rate (%)	Coefficient of variation (%)
0 (blank)	39	—	3.7
3	43	133	3.5
5	44	100	3.7
10	50	110	5.0
50	88	98	5.4
100	135	96	5.6
250	282	97	4.0
500	546	101	4.2

[a] Mean of three independent tests.

Table 4—Recovery of urea from white wine

Urea added (µg/L)	Urea found[a] (µg/L)	Recovery[a] rate (%)	Coefficient of variation (%)
0 (blank)	24	—	5.3
3	28	133	5.1
5	29	100	4.9
10	34	100	5.1
50	72	96	4.9
100	129	105	4.2
250	273	100	3.9
500	508	97	5.6

[a] Mean of three independent tests.

96 to 110% in red wine and from 96 to 105% in white wine. The improved method provides higher sensitivity for detection of low concentrations of urea with a lower limit of ≈ 5 µg/L.

CONCLUSIONS

THE APPROPRIATE SELECTION of eluent, column, and chiral derivatives for HPLC detection of urea in solution provided the high sensitivity necessary for accurate measurements at low concentrations. Our method can detect urea as low as 5 µg/L in

Stability of Metabolically Conjugated Precursors of Meat and Milk Flavor Compounds in Various Solvents

L.-H. HAN and R.C. LINDSAY

ABSTRACT

The stability of selected metabolic conjugates (phenylglucuronide, phenylphosphate, and naphthylsulfate) was determined in model systems composed of water and various ratios (3:1, 1:1, 1:3) of selected solvents (hexane, chloroform, ethyl acetate, diethyl ether, or methanol) held at either 22°C or 40°C for 30 min under various pH conditions (pH 1.5, 3.2, or 6.5). Notable hydrolysis occurred only for the more polar solvents held in contact with acidic aqueous phases. Conditions were identified for minimizing hydrolysis of conjugates during extraction of fat and free alkylphenols from milk and meat products with diethyl ether. They were pH near neutral, short exposure time, near ambient temperature, presence of excess water, and saturation of aqueous phase with sodium chloride.

Key Words: milk flavor, precursors, metabolic conjugates, solvent stability

INTRODUCTION

SEVERAL VOLATILE ALKYLPHENOLS have been identified in meats (Ha and Lindsay, 1991a; Lorenz et al., 1983; Nixon et al., 1979), butter (Urbach et al., 1972), cheeses (Ha and Lindsay, 1991b, c; Moins et al., 1976; Ramshaw, 1985) and off-flavored fish (Heil and Lindsay, 1988). Alkylphenols often exhibit low flavor thresholds (Ha and Lindsay, 1991a; Urbach et al., 1972; Wasserman, 1966), and these compounds have been reported to provide influential flavors to meats, cheeses and butter (Ha and Lindsay, 1991a, b, c; Urbach et al., 1972). Metabolically conjugated alkylphenols were found in milk by early researchers (Brewington et al., 1973, 1974). Lopez and Lindsay (1993a) found that conjugates of alkylphenols were precursors to cowy and sheep-like flavor compounds in skimmilks. Concentrations of alkylphenols also increased during cooking of mutton which led to the hypothesis that thermally labile precursors for alkylphenols were present in meat (Brennand and Lindsay, 1992).

Metabolic conjugates in aqueous systems of biological origin have been isolated and quantified (Capel et al., 1972, 1974; Kao et al., 1979; Porteus and Williams, 1949; Stehly and Hayton, 1988). Free alkylphenols have been isolated using steam distillation extraction (Brennand and Lindsay, 1992; Ha and Lindsay, 1991a, b, c; Heil and Lindsay, 1988). However, attempts to analyze individual alkylphenols and their conjugates in complex fatty foods has been difficult. Lopez and Lindsay (1993a) analyzed alkylphenols and corresponding conjugates in skimmilk, but did not analyze these compounds in whole milk because of interferences from milkfat.

Thus, because of interference from fats, fatty foods have not been analyzed directly for alkylphenol conjugates. It has been necessary to devise means for defatting samples before applying adsorptive or other techniques for isolation of intact conjugates (Lopez and Lindsay, 1993a). Similarly, analysis of free alkylphenols in fatty foods requires their separation from bound conjugate forms before distillation or extraction to isolate them from fats in which they are dissolved (Han and Lindsay, 1995).

Extraction of fats from fatty foods with solvents is the most direct means for facilitating analysis of free alkylphenols and their conjugates. However, the stability of conjugates in contact

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with such solvents is questionable. Lopez and Lindsay (1993b) have reported instability of alkylphenol conjugates when they were placed in contact with certain organic solvents, similar to other reports (Burstein and Lieberman, 1958; Burstein et al., 1960). Our objectives were to determine the stability of phenolic conjugates when placed in contact with selected solvents, and to identify conditions to help develop analytical procedures that would not distort quantitative relationships between free and conjugated forms of alkylphenols from fatty foods.

MATERIALS & METHODS

Reagents and chemicals

Phenyl β-D-glucuronide, potassium 2-naphthylsulfate and tetrabutylammonium hydrogen sulfate (TBAHS) were purchased from Sigma Chemical Co. (St. Louis, MO). Disodium phenylphosphate, p-cresol and phenol were purchased from Aldrich Chemical Co. (Milwaukee, WI). Acetonitrile, hexane, chloroform, methanol and ethyl acetate were HPLC grade from Fisher Scientific (Fair Lawn, NJ). Reagent grade sulfuric acid, sodium sulfate anhydrous, sodium chloride and diethyl ether were obtained from Mallinckrodt (Rochester, NY). Diethyl ether was single-plate distilled in a glass apparatus before use. Tween 80® was obtained from ICI (Wilmington, DE). Butteroil was obtained from the Center for Dairy Research, UW-Madison, WI.

Analytical methods

A high-performance liquid chromatography (HPLC) system consisting of an ISCO Model 2350 pump (Lincoln, NE) and a C_{18} reverse-phase column (Zorbax ODS 4.6 i.d. × 25 cm, DuPont, Wilmington, DE) with an ISCO variable wavelength absorbance detector set at 210 nm was employed. Chromatograms were recorded with a Model 4100 computing integrator (Spectra-Physics, San Jose, CA). A 10-μL sample loop was used for sample introduction. An isocratic paired-ion mobile phase composed of 7 mM TBAHS in acetonitrile and water (40:60) was pumped at 1.5 mL/min, and the recorder chart speed was 1 cm/min (Frausson et al., 1976; Knox and Hurand, 1977; Lopez and Lindsay, 1993a; Ragan and Mackinnon, 1979; Sawa et al., 1988). All samples and the mobile phase were passed through a 0.2-μm Teflon filter (Alltech, Deerfield, IL) before injection. The mobile phase was degassed under reduced pressure (100 mm Hg) and held in an ultrasonic bath (Heat Systems-Ultrasonics Inc., Plainview, NY) for 15 min before use.

Stability of phenolic conjugates

Bulky solvent/water systems. Phenyl β-D-glucuronide, potassium 2-naphthylsulfate and disodium phenylphosphate standards were dissolved individually either in twice-distilled water (pH 6.5), or similar water which had been adjusted to either pH 1.5 or pH 3.2 with 2 N sulfuric acid. Solutions of conjugates (10 mL; 100 μg/mL) were pipetted into 50-mL test tubes (25 × 150 mm, screw-cap with PTFE-faced rubber liner), and then were mixed individually with either 3 mL, 10 mL or 30 mL of a selected solvent (hexane, chloroform, ethyl acetate, diethyl ether or methanol). Sample preparations were hand-shaken vigorously for 30 sec, and then placed in a shaker water bath (Model 406015, Cambridge Instruments, Inc., Buffalo, NY) at 40°C (22°C for diethyl ether) for 30 min prior to analysis. Organic solvents were removed by evaporation under a slow stream of nitrogen at 25°C, and 10 μL of p-cresol stock solution (0.1 mg/μL methanol; 1 mg total) was added as a reference standard to the remaining aqueous solutions. Samples of aqueous solutions were then analyzed for conjugates by HPLC, and differences in concentrations between original stock solutions and experimental samples were calculated before computing % recoveries.

Table 1—Stability of phenylglucuronide during brief exposure to several solvent-water systems

Solvent system	Recovery (%)[a]		
	pH 1.5	pH 3.2	pH 6.5
Water : Hexane (40°C for 30 min)			
1 : 3	100 ± 0.0[b]	101 ± 0.0	100 ± 0.7
1 : 1	101 ± 0.2	98 ± 0.8	102 ± 0.4
3 : 1	100 ± 0.0	94 ± 1.4	100 ± 0.0
Water : Chloroform (40°C for 30 min)			
1 : 3	100 ± 0.0	97 ± 1.1	101 ± 0.5
1 : 1	102 ± 1.3	98 ± 0.6	98 ± 0.6
3 : 1	99 ± 0.0	98 ± 0.4	100 ± 0.5
Water : Ethyl acetate (40°C for 30 min)			
1 : 3	93 ± 2.8	94 ± 0.8	97 ± 0.8
1 : 1	95 ± 0.6	92 ± 1.7	97 ± 0.4
3 : 1	95 ± 0.4	91 ± 2.9	103 ± 0.3
Water : Diethyl ether (22°C for 30 min)			
1 : 3	98 ± 1.4	97 ± 0.7	97 ± 0.4
1 : 1	97 ± 1.4	97 ± 0.6	99 ± 0.0
3 : 1	97 ± 0.1	100 ± 0.1	98 ± 0.0
Water : Methanol (40°C for 30 min)			
1 : 3	92 ± 3.5	93 ± 1.3	95 ± 0.8
1 : 1	95 ± 1.5	97 ± 1.6	98 ± 0.1
3 : 1	95 ± 0.8	100 ± 1.7	103 ± 0.0

[a] Initial concentration = 100 µg/mL H_2O.
[b] Mean ± standard deviation for duplicate sample analyses.

Table 2—Stability of phenylphosphate during brief exposure to several solvent-water systems

Solvent system	Recovery (%)[a]		
	pH 1.5	pH 3.2	pH 6.5
Water : Hexane (40°C for 30 min)			
1 : 3	94 ± 1.3[b]	97 ± 0.1	95 ± 0.3
1 : 1	93 ± 2.0	96 ± 0.1	100 ± 0.1
3 : 1	95 ± 0.7	97 ± 0.0	97 ± 0.1
Water : Chloroform (40°C for 30 min)			
1 : 3	98 ± 0.1	101 ± 0.0	101 ± 0.3
1 : 1	100 ± 0.0	100 ± 0.0	99 ± 0.0
3 : 1	98 ± 0.1	100 ± 0.1	100 ± 0.0
Water : Ethyl acetate (40°C for 30 min)			
1 : 3	74 ± 1.2	76 ± 3.5	94 ± 1.7
1 : 1	80 ± 0.1	76 ± 1.3	96 ± 1.3
3 : 1	84 ± 1.1	87 ± 2.1	102 ± 0.1
Water : Diethyl ether (22°C for 30 min)			
1 : 3	89 ± 1.3	92 ± 2.2	97 ± 0.4
1 : 1	90 ± 0.7	92 ± 1.8	97 ± 0.7
3 : 1	93 ± 0.7	93 ± 0.4	100 ± 0.0
Water : Methanol (40°C for 30 min)			
1 : 3	84 ± 3.3	83 ± 1.4	94 ± 1.3
1 : 1	96 ± 1.7	96 ± 0.8	92 ± 1.4
3 : 1	95 ± 1.1	94 ± 0.8	95 ± 0.8

[a] Initial concentration = 100 µg/mL H_2O.
[b] Mean ± standard deviation for duplicate sample analyses.

Stability of conjugates in diethyl ether. Samples of conjugates (phenylglucuronide, phenylphosphate, naphthylsulfate; 2 mg each) were added into either 40 mL of anhydrous diethyl ether or 40 mL of diethyl ether containing 1% water (pH 6.5 or pH 1.5). Anhydrous diethyl ether was prepared by adding excess anhydrous sodium sulfate, and storing the diethyl ether in the dark for 24 hr. Water (1%) was introduced into anhydrous diethyl ether (40 mL) by adding 400 µL of the appropriate pH-adjusted water, and then rapidly blending the mixture with a high-speed Polytron homogenizer (Brinkmann Instruments Co., Westbury, NY) for 30 sec.

Samples were then quickly placed in an ultrasonic water bath (Heat Systems-Ultrasonics Inc., Plainview, NY) and held at 22°C for 30 min to aid in maintaining dispersions. After storing, the ether-based solutions were combined with 20 mL water, and then diethyl ether was evaporated under a slow stream of nitrogen. A reference standard (p-cresol; 20 µL; 2.0 mg; 0.1 mg/µL methanol) was added to each aqueous solution, and the concentrations of intact conjugate compounds were determined by HPLC analysis.

Effect of sodium chloride on partitioning of phenolic conjugates into diethyl ether. Sodium chloride (1, 2, 3, or 4g) was added to so-lutions (10 mL each) of a conjugate mixture (100 µg/mL) to produce serial aqueous solutions containing 10, 20, 30 or 40% sodium chloride. Diethyl ether (10 mL) was added to each sample, and they were shaken by hand for 30 sec. Then, after storing 30 min, each diethyl ether phase was recovered and added to a new 10-mL aliquot of the aqueous solution containing the mixture of conjugates (100 µg/mL each). This was necessary to increase the total conjugate concentration in a sample to a range detectable by HPLC. The diethyl ether was evaporated under a slow stream of nitrogen, and p-cresol was added as a reference standard (1.0 mg; 10 µL; 0.1 mg/µL methanol). HPLC analysis was then conducted, and amounts of conjugates in the diethyl ether phase were determined by difference between corresponding analytical data for basic stock solution and the stock solution plus that from the diethyl ether extracts.

Stability of conjugates in water and fat emulsion systems. Simulated cream and butter emulsion systems were prepared by emulsifying water and anhydrous milkfat (Center for Dairy Research, UW-Madison, WI) in the ratios of 50:40 (cream) or 20:80 (butter). Tween 80® (3%) was added as an emulsifier, and 2 mg of each of the conjugates (phenylglucuronide, phenylphosphate, naphthylsulfate) were dispersed in each of the cream and butter systems (20g each). Samples were then incubated at 40°C for either 30 min or 24 hr, or were heated at 90°C in a water bath for either 30 min or 2.5 hr.

Samples were then combined with 10 mL distilled water and shaken with 20 mL diethyl ether to remove milkfat. Then aqueous residues were each again combined with 10 mL distilled water and extracted with another 10 mL diethyl ether before centrifuging (6000 × g; Sorvall refrigerated centrifuge, DuPont Inst., Des Plaines, IL) at 10°C for 20 min to complete removal of fat. Final aqueous samples containing conjugates were collected, and p-cresol was added as a reference standard (20 µL; 2.0 mg; 0.1 mg/µL methanol) before analysis by HPLC.

RESULTS & DISCUSSION

Stability of conjugates in bulky water:solvent systems

Stabilities of aqueous solutions of phenylglucuronide, (Table 1) phenylphosphate (Table 2) and naphthylsulfate (Table 3) to various solvents that might be employed in defatting or extractions of free alkylphenols from fatty foods were compared. This series of trials was carried out to evaluate effects of bulky solvent exposure conditions that might affect the degree of hydrolysis of conjugates during extractions. This provided guidance in selection of extraction conditions which would not distort quantitative relationships between free and conjugate-bound phenols in fatty foods.

The brief 30-min exposure was used because it represented our estimated solvent contact time for conjugates dissolved in the water phase of foods during extractions. Similarly, the exposure temperature of 40°C was used for most systems because it might be used to liquefy animal fats to improve extraction efficiencies. However, in the case of diethyl ether, exposure temperature was maintained at 22°C to minimize evaporation of solvent. The ratios of water to solvents were varied for each system (3:1, 1:1, 1:3) to determine whether the relative bulk amounts of water present in a system affected the degree of hydrolysis.

In these studies, residual phenolic conjugates were quantitatively measured to determine the degree of hydrolysis and % recoveries. However, although not quantified, the corresponding free phenols were consistently noted during HPLC analysis which confirmed that hydrolysis of conjugates had occurred. Thus, we concluded that conjugates were not simply unrecovered during experimental manipulation.

Exposure of aqueous solutions of the three conjugates at pH 6.5 to the solvents resulted in comparatively little hydrolysis. This was especially the case for phenylglucuronide where recovery was >95% from pH 6.5 systems for all solvent combinations (Table 1). Phenylphosphate (Table 2) and naphthylsulfate (Table 3) were hydrolyzed slightly at pH 6.5 (3–8%) when in contact with the more polar solvent systems, and this was more notable in methanol:water systems. It has been reported (Burstein and Lieberman, 1958) that conjugates were susceptible to hydrolysis when water was present and they were dispersed in non-ionizable solvent systems.

Table 3—Stability of naphthylsulfate during brief exposure to several solvent-water systems

Solvent system	Recovery (%)[a]		
	pH 1.5	pH 3.2	pH 6.5
Water : Hexane (40°C for 30 min)			
1 : 3	91 ± 0.1[b]	96 ± 0.8	99 ± 0.0
1 : 1	92 ± 1.0	93 ± 0.5	101 ± 0.0
3 : 1	92 ± 0.7	91 ± 0.5	100 ± 0.0
Water : Chloroform (40°C for 30 min)			
1 : 3	73 ± 1.1	76 ± 1.6	101 ± 0.1
1 : 1	73 ± 0.7	78 ± 0.5	99 ± 0.1
3 : 1	84 ± 1.3	80 ± 1.1	100 ± 0.7
Water : Ethyl acetate (4C°C for 30 min)			
1 : 3	48 ± 2.6	76 ± 3.1	94 ± 0.3
1 : 1	79 ± 2.2	76 ± 4.2	96 ± 0.1
3 : 1	87 ± 2.0	86 ± 0.7	96 ± 0.1
Water : Diethyl ether (22°C for 30 min)			
1 : 3	87 ± 1.1	86 ± 0.3	96 ± 0.3
1 : 1	87 ± 0.8	87 ± 0.7	97 ± 1.1
3 : 1	92 ± 1.4	97 ± 1.0	97 ± 0.7
Water : Methanol (40°C for 30 min)			
1 : 3	69 ± 3.2	78 ± 2.0	92 ± 0.0
1 : 1	83 ± 2.8	83 ± 1.6	98 ± 0.1
3 : 1	87 ± 1.8	94 ± 2.2	97 ± 0.1

[a] Initial concentration = 100 µg/mL H_2O.
[b] Mean ± standard deviaticn for duplicate sample analyses.

The data for solutions at pH 6.5 which were contacted with either hexane or chloroform showed that the recovery of the conjugates was nearly 100%. Presumably, hydrolysis did not occur because of low solubility of the conjugates in the nonpolar solvents. With ethyl acetate and diethyl ether in contact with pH 6.5 water, the slightly higher polarities probably led to some solution and hydrolysis of conjugates (<6%), especially for phenylphosphate (Table 2) and naphthylsulfate (Table 3).

The effects of acidic conditions on stability of conjugates in the water:solvent systems was studied using aqueous systems at pH 1.5 or 3.2. Phenylglucuronide contacted with hexane or chloroform gave similar results to the pH 6.5 systems, nearly 100% recovery. In the presence of the more polar solvents, only slight (<8%) hydrolysis of phenylglucuronide occurred. Overall phenylglucuronide was most resistant to hydrolysis regardless of conditions.

Little hydrolysis of phenylphosphate and naphthylsulfate occurred under acidic conditions when these conjugates were contacted with hexane (Tables 2 and 3). However, while phenylphosphate was quite stable during chloroform exposure under acidic conditions, naphthylsulfate was hydrolyzed to a notable degree (16–27%; Table 3). Similarly, acidic conditions increased hydrolysis of these two conjugates when they were contacted with more polar solvents.

Acidic conditions in two-phase systems may suppress ionization and increase the partitioning of conjugates into the organic solvent phase where the solvent aids in the hydrolysis (Burstein and Lieberman, 1958). In two-phase systems, hydrolysis of conjugates in organic media follows first-order reaction kinetics and occurs in two stages (Burstein et al., 1960; Marsh, 1966). The first stage involves partitioning of conjugate into the organic phase, followed by solvolysis of conjugate in the nonionizing solvent.

The amount of water affects the rate of hydrolysis of conjugates, and this has an important effect on the rate-determining step of conjugate solvolysis (Burstein et al., 1960). In our studies, the amount of water was varied for each water:solvent system (1:3, 1:1, 3:1), and the concentration of conjugate in each aqueous solution (100 µg/mL) remained constant. Effects related to the amount of bulk water on the degree of hydrolysis were not observed in all trials. However, such effects were found in most instances where notable (>10%) overall hydrolysis occurred (Tables 2 and 3). In the case of naphthylsulfate exposure to water:ethyl acetate (Table 3), 52% was hydrolyzed in the 1:

3 system, 21% in the 1:1 system, and 13% in the 3:1 system. The amount of bulk water was not highly restrictive. We concluded that the higher concentration of conjugate in the water portion of 1:3 water:solvent systems caused a greater partitioning of conjugate into the organic phase. As a result, the solvolysis step was accelerated and more hydrolysis occurred than for samples containing more dilute concentrations of conjugates in the aqueous phase.

Because of its low polarity, though it is reasonably efficient for extracting fats, hexane provided inadequate extraction of phenol and other polar phenolic analogs from aqueous systems (Han and Lindsay, 1995). The higher boiling points for the more polar solvents, chloroform, methanol and ethyl acetate, caused difficulties with hydrolysis and solvent removal. The coextraction of water and non-volatile constituents with methanol also caused additional problems in determination of free alkylphenols.

Thus, of solvents in the two-phase systems, diethyl ether was the most promising for extraction of fats and free alkylphenols from fatty foods. Several researchers have reported that higher recoveries of phenol and its alkyl derivatives were obtained with diethyl ether than with other solvents when applied in two-phase extractions (Hrivnak and Steklac, 1984; Jones et al., 1993; Kao et al., 1979; Tyman et al., 1989). Stehly and Hayton (1988) reported that pentachlorophenol could be removed from aqueous samples using diethyl ether extraction without hydrolysis of glucuronide and sulfate conjugates which remained in the aqueous phase. However, extractions with either ethyl acetate or butanol resulted in only partial recovery of conjugates, and some hydrolysis occurred at pH 2 and 8. Lopez and Lindsay (1993b) also reported that alkylphenol conjugates held in the presence of ethyl acetate at 40°C for 30 min were unstable.

Lieberman and Dobriner (1948) reported that sulfate conjugates were not appreciably hydrolyzed in the presence of diethyl ether when extractions were carried out at room temperature and pH of the aqueous phase was >1.0. Results of our earlier bulky water:solvent trials had shown that phenylglucuronide was essentially stable under the trial conditions of contact (22°C for 30 min) with diethyl ether for all three pH values (1.5, 3.2, 6.5; Table 1). Similarly, phenylphosphate and naphthylsulfate were quite stable when contacted with diethyl ether at pH 6.5 (Table 2 and 3). However, under acidic conditions (<pH 3.2), some hydrolysis for the two conjugates occurred (>13%). Some limited effects of the amount of bulk phase water on conjugate hydrolysis were also noted for diethyl ether. In the trial for naphthylsulfate at pH 3.2 (Table 3) the lower water contents (1:3 and 1:1) resulted in about 10% more hydrolysis than with the higher water content (3:1, water:diethyl ether).

Effects of restricted water contact. The effects of exposing conjugates to very limited amounts of water (1%) in diethyl ether systems was also investigated (Table 4). Dispersion and exposure of conjugates to anhydrous diethyl ether resulted in >90% recovery of each of the three conjugates, and under anhydrous conditions phenylphosphate was least stable. However, when 1% water (pH 6.5 or 1.5) was added to the diethyl ether, considerable hydrolysis occurred for each of the conjugates except for phenylglucuronide at pH 6.5 (Table 4).

The hydrolytic effect of low water concentrations was especially pronounced at the lower pH (1.5) for phenylphosphate and naphthylsulfate where all of the conjugate was hydrolyzed. Hydrolysis was confirmed in these systems because the corresponding free phenolic substance was also found in the analysis. The rapid hydrolysis was probably caused by the strong partitioning of conjugates into diethyl ether from the aqueous phase where they were concentrated because of the limited amount of water.

Effects of contact with sodium chloride. Elevated concentrations of sodium chloride in the aqueous phase of samples provided strong salting-out effects which resulted in increased extraction recovery of phenol and phenolic substances (Jones et al., 1993). Effects of adding sodium chloride to the aqueous

Table 4—Effect of restricted amount of water on the stability of selected phenolic conjugates exposed to diethyl ether for 30 min at 22°C

| Sample treatment | pH | Recovery (%)[a] | | |
		Phenyl glucuronide	Phenyl phosphate	Naphthyl sulfate
Diethyl ether				
anhydrous	—	97.3 ± 2.4	90.6 ± 1.0	95.9 ± 3.1
+ 1% water	6.5	98.5 ± 1.7	68.3 ± 3.6	12.2 ± 4.7
+ 1% water	1.5	74.6 ± 4.5	trace	nd[b]

[a] Each sample initially contained 2 mg of each conjugate dispersed in 40 mL of diethyl ether; Mean ± standard deviation for duplicate sample analyses.

[b] Not detected.

Table 5—Effect of sodium chloride concentration on the partitioning of selected phenolic conjugates into diethyl ether from aqueous solutions

| NaCl conc in aqueous phase (%) | Amount of conjugate partitioned into diethyl ether (%)[a] | | |
	Phenyl glucuronide	Phenyl-phosphate	Naphthyl-sulfate
0	1.3 ± 0.18[b]	1.5 ± 0.10	1.9 ± 0.07
10	0.6 ± 0.07	0.6 ± 0.00	2.0 ± 0.02
20	0.2 ± 0.05	0.4 ± 0.02	1.5 ± 0.03
30	0.8 ± 0.05	0.5 ± 0.02	2.1 ± 0.09
40 (saturated)	0.7 ± 0.07	0.9 ± 0.05	1.8 ± 0.06

[a] Initial concentration = 1 mg/10 mL aqueous sample.

[b] Mean ± standard deviation for duplicate sample analysis.

phase upon partitioning of phenolic conjugates to diethyl ether were compared (Table 5). Only conjugates were found in the diethyl ether, and free phenolic substances were not detected which indicated that hydrolysis in this system was very limited.

Generally, the addition of sodium chloride (10–40%) to the aqueous phase decreased the amount of conjugate in the diethyl ether phase, but this did not appear to apply for naphthylsulfate where no effect was observed. It was not possible to directly analyze samples containing sodium chloride by HPLC because of the salt. As a result, the near threshold-detection amounts of conjugates extracted into diethyl ether were combined with those in fresh stock solutions before analyses.

Overall, the presence of sodium chloride at elevated ionic strengths generally did not induce hydrolysis. It enhanced the solubility of conjugates in the aqueous phase while suppressing their partitioning into the diethyl ether. Thus, the use of sodium chloride in extractions not only should increase recovery of free alkylphenols, but also should reduce errors caused by solution of conjugates in diethyl ether.

Stability of conjugates in simulated cream and butter systems

Fats are esters of glycerol and fatty acids, and hence may exert hydrolytic effects on phenolic conjugates similar to those from certain solvents. Trials with simulated butter (80:20, fat: water) and cream (40:60, fat:water) (Table 6) showed relatively little hydrolysis occurred at 40°C during exposure for 0.5 hr. Some hydrolysis (<15%) was observed when samples containing the more labile phenylphosphate and naphthylsulfate were held for 24 hr. Again, phenylglucuronide was the most stable.

In most instances heating of simulated cream and butter systems at 90°C resulted in some hydrolysis within 0.5 hr (<16%). However, when heating time was extended to 2.5 hr, hydrolysis (>17%) was noted for all conjugates. Under the higher heating conditions, phenylphosphate was nearly all hydrolyzed within 2.5 hr. Comparison with results of Lopez and Lindsay (1993b), who studied the stability of phenolic conjugates in purely aqueous systems, indicated that presence of vegetable oil may have contributed to hydrolysis of phenylphosphate in their studies. Thus, hydrolysis of conjugates in fatty food systems may be accelerated compared to purely aqueous systems. Fats may enhance the rate of flavor development by this mechanism in foods of animal origin when they are heated as in roasting mutton (Brennand and Lindsay, 1992).

CONCLUSIONS

CONDITIONS for isolation of conjugates which would be expected to give negligible hydrolysis are the use of diethyl ether as extractant at 22°C with exposure time of 30 min or less. Avoidance of heating fatty foods, saturation of aqueous phase with sodium chloride and adjusting to pH 6.5 for extraction of the fats and free alkylphenols would minimize potential distortion of quantitative relationships between free and bound forms alkylphenols.

Table 6—Stability of selected phenolic conjugates in model cream and butter systems

| Sample treatments (°C) | (hr) | Recovery (%)[a] | | |
		Phenyl-glucuronide	Phenyl-phosphate	Naphthyl-sulfate
Cream				
40	0.5	98 ± 1.0[b]	97 ± 0.3	87 ± 2.6
	24	97 ± 0.5	91 ± 1.1	89 ± 1.7
90	0.5	96 ± 0.7	88 ± 0.9	87 ± 3.2
	2.5	83 ± 1.3	14 ± 2.8	79 ± 3.6
Butter				
40	0.5	97 ± 0.4	94 ± 1.6	88 ± 2.0
	24	95 ± 0.4	85 ± 1.0	87 ± 2.8
90	0.5	95 ± 0.5	84 ± 0.7	88 ± 1.9
	2.5	83 ± 0.9	8 ± 6.3	74 ± 6.2

[a] Initial concentration of each conjugate was 2 mg/20g sample.

[b] Mean ± standard deviation for triplicate sample analyses.

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Hydrolysates from Proteolysis of Heat-denatured Whey Proteins

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ABSTRACT

Whey protein isolate was denatured at 85°C. pH 4.6 for 30 min to produce heat denatured whey protein isolate (HDWPI) which was hydrolyzed with trypsin, chymotrypsin, Alcalase or Neutrase to 2.8, 4.3, 6.0 or 8.0% degree of hydrolysis (DH). Analysis of freeze-dried fractions revealed a linear increase in primary amino groups, non-protein nitrogen and ash contents. Polyacrylamide gel electrophoresis showed that high and intermediate molecular weight peptides were converted to lower molecular weights with progress of hydrolysis. Differences in proteolysis patterns were observed with different enzymes. The time required to achieve equivalent hydrolysis at 1, 2, 3 or 4% enzyme/substrate ratio varied with the type of enzyme and DH.

Key Words: whey protein, heat denatured, hydrolysates, amino groups

INTRODUCTION

PROTEINS FROM CHEESE WHEY can be recovered by heat insolubilization, filtration, ion exchange or chemical precipitation (Matthews, 1984). Heat precipitation is preceded by denaturation of the proteins and changes in protein conformation. Heat denaturation of whey protein is a two-stage process involving unfolding at moderate heat treatments followed by aggregation when subjected to more severe heat treatments (deWit and Klarenbeek, 1984; Schmidt et al., 1984). Denaturation is affected by environmental conditions, such as pH, calcium, and solids concentration. Whey proteins are more heat labile than caseins and the decreasing order of heat sensitivity of whey protein fractions is immune globulins, bovine serum albumin, β-lactoglobulin, and α-lactalbumin (Fox, 1981). The pattern of heat denaturation of the major whey protein fractions, β-lactoglobulin and α-lactalbumin, in cheese whey paralleled that in skim milk (Hillier and Lyster, 1979). The heat-induced changes in whey proteins are expected to alter their functionality. Heating at neutral pH was hypothesized to enhance emulsification and foaming while heating at an acidic pH should result in high viscosity, lower gel temperature and high water absorption (Schmidt et al., 1984).

Functionality of milk protein can also be manipulated by chemical or enzymatic modification. Chemical modification by succinylation or acetylation of the ε-amino group was reported by Kinsella and Shetty (1979) or amidation, esterification, phosphorylation and thiolation have also been reported (Kester and Richardson, 1984; Vojdani and Whitaker, 1994). Chemical modification altered nutritional value (Matoba et al., 1980) and also increased the resistance of adjacent peptide bonds to peptic and tryptic hydrolysis (Poncz and Dearborn, 1983). Enzymatic modification of whey proteins using food grade proteolytic enzymes is a promising approach to alter protein functionality (Kuehler and Stine, 1974; Kester and Richardson, 1984; Vojdani and Whitaker, 1994). Proteolysis patterns of heat denatured whey proteins by different proteases may differ from those of native proteins. Our objective was to study the preparation and composition of hydrolysates from proteolysis of heat-denatured whey protein.

MATERIALS & METHODS

Preparation of heat denatured whey protein isolate

Commercial whey protein (Kerry Foods Inc., Chicago, IL) was reconstituted at 20% in distilled water at 43°C and stirred for 1 hr. The dispersion was concentrated by ultrafiltration on an Abcor SS1 (Koch Membrane, Wilmington, MA) spiral-wound with a molecular weight cut-off of 10,000. The dispersion was concentrated two-fold and diafiltered by repeated washing with distilled water till the permeate was clear. The washed retentate was adjusted to pH 4.6 with 2N HCl, heated to 85°C for 30 min in a steam-jacketed kettle and stored overnight at 4°C. The clear supernatant was decanted and the sediment was freeze-dried and designated as Heat Denatured Whey Protein Isolate (HDWPI).

Enzymatic hydrolysis

Bovine trypsin and α-chymotrypsin (Sigma Chemical Co., St. Louis, MO); Alcalase and Neutrase (Novo Laboratories, Wilton, CT) were used for hydrolysis. Trypsin (44.07 Anson units) and α-chymotrypsin (18.93 Anson units) were used at 25°C and at pH 7.6 and pH 7.8, respectively; while Alcalase (6.28 Anson units) and Neutrase (2.81 Anson units) were used at 50°C and pH 8.5 and 7.5, respectively. One Anson unit is the amount of enzyme necessary to cause an increase in absorbance of 0.001/min under conditions of the assay. The assay of proteolytic activity (Anson, 1938) was modified to replace the substrate, hemoglobin, with 2.5% HDWPI.

Inactivation conditions for the 4 enzymes were determined in 4 mg/mL aqueous solution diluted to 0.2 mg/mL with 0.5 M potassium phosphate buffer or 0.1M HCl. The solutions were adjusted to pH values between 2.0 and 12.0 at 0.5 increments. In a cuvette 2 mL of the enzyme solution was placed and its temperature was increased at 1°C/min. Absorbance at 280 nm was recorded as a function of temperature on a Gilford Model 250 spectrophotometer (McCord and Kilara, 1982). The temperature, time, and pH corresponding to mid-points of the inflection were taken as optimum conditions for inactivation. Enzyme inactivation was confirmed by testing residual activity on 10% (w/v) HDWPI solution at 4% enzyme: substrate ratio and an assay time of 10 min and comparing with controls with active enzyme.

Varying enzyme concentration at constant substrate

A 10% w/v aqueous protein dispersion was used to test the effects of enzyme concentration. Optimum conditions for enzyme activity were-trypsin (25°C, pH 7.6); chymotrypsin (25°C, pH 7.8); Alcalase (50°C, pH 8.5), and Neutrase (50°C, pH 7.5). Enzyme:substrate ratio was varied between 1 and 4% in terms of equivalent Anson units. A total of 10 mL of the substrate was used and the hydrolysis was carried out at the optimum temperature for each enzyme in a water bath. The pH was maintained at optimum for each enzyme by 0.1 N NaOH and the volume of base consumed at 10 min intervals was recorded over 60 min.

Varying substrate concentration at constant enzyme concentration

Substrate concentration was varied to achieve constant E/S ratios. Two levels of E/S concentration, (2 and 4%) were investigated at each of three substrate concentrations, (6, 8, and 10%). Conditions of hydrolysis were similar to those described.
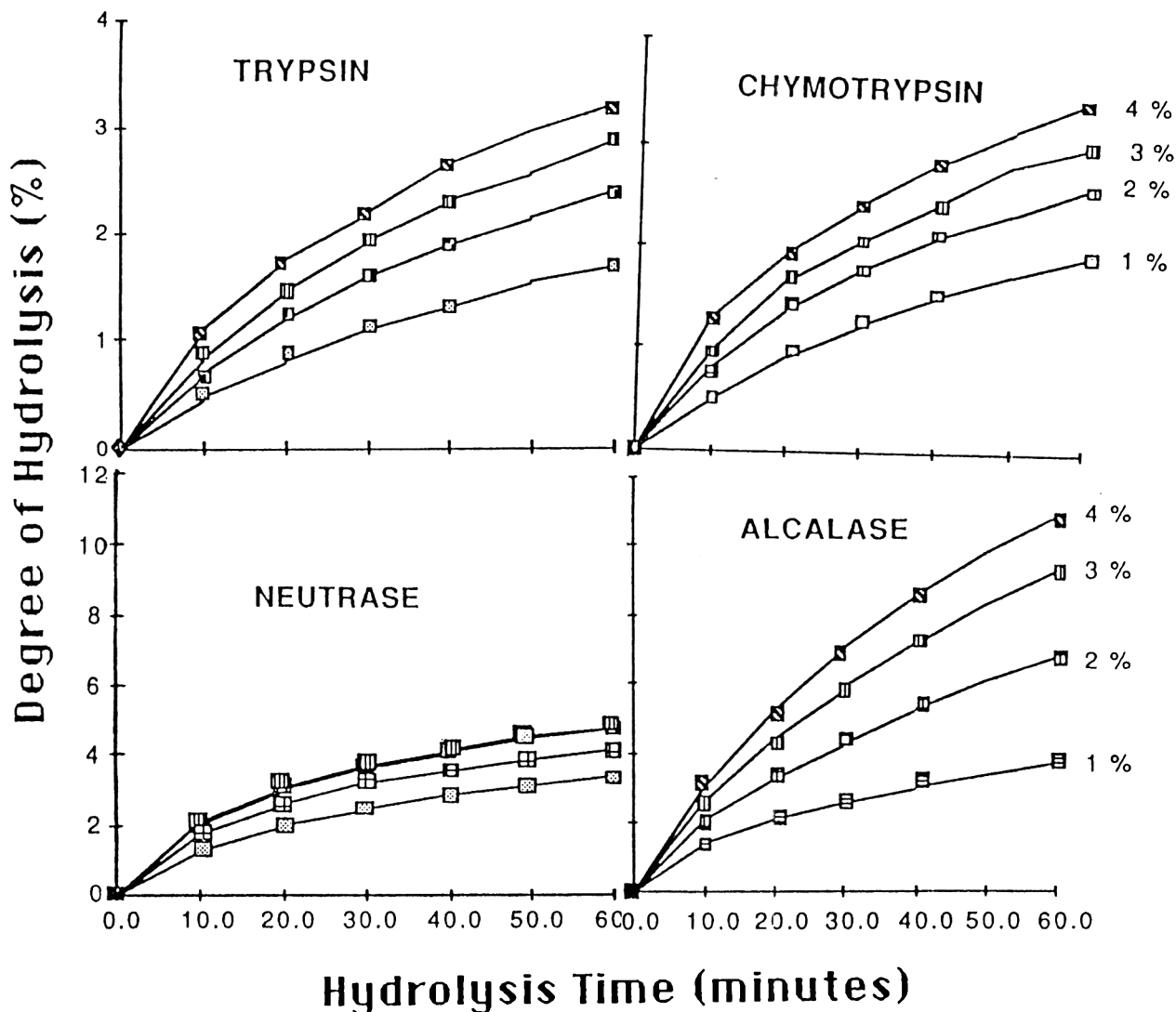
Fig. 1—Hydrolysis curves of heat-denatured whey protein isolate as related to enzyme: substrate ratio and reaction time.

Preparation of hydrolysates

The degree of hydrolysis (DH) was controlled as described by the equation reported by Adler-Nissen (1976, 1986)

$$D\,H = \frac{B \times N_b}{MP} \times \frac{1}{\alpha} \times \frac{1}{H_{tot}} \times 100$$

where B = base consumption, N_b = normality of the base, α = degree of dissociation of the amino group, MP = mass of protein and H_{tot} = total number of peptide bonds in the substrate. The degree of dissociation of the amino groups, alpha, is expressed as

$$\alpha = \frac{10^{pH - pK}}{1 + 10^{pH - pK}}$$

where the pH is the value at which proteolysis was conducted, pK = 7.7 at 25°C and 7.1 at 50°C. The parameter H_{tot} = 8.8 meq/kg. From this equation the base consumption required to achieve the required DH was calculated. Hydrolysis was allowed to continue until the predicted base consumption was attained before initiating enzyme inactivation. A control was treated under similar conditions but without the added enzyme.

Hydrolysis was carried out in a total reaction volume of 2 L of a 5% protein (w/v) solution. The DH was calculated by correcting the base consumption for the blank. Inactivation conditions (determined for each enzyme) were used to terminate enzyme activity, before readjusting the pH to 7.0 and freeze-drying.

Proximate composition

Moisture, fat, protein (N × 6.38), ash and nitrogen-free extract were determined using standard methods (AOAC, 1980). Mineral analysis was performed at the Poultry Science Department at the Pennsylvania State University. For estimation of Na, K, Ca, and Mg, samples were ashed, dissolved in distilled water and analyzed on an atomic absorption spectrophotometer.

Nonprotein nitrogen (NPN) determination

A 0.5% (w/v) protein dispersion (15 mL) was adjusted to a final 12% tricholoroacetic acid (TCA) and stirred for 5 min before filtering (Whatman No. 1). Nitrogen content was estimated in a 2 mL aliquot of the filtrate using a semi-micro Kjeldahl procedure. Nitrogen content was calculated and expressed as percentage of total protein nitrogen.

Primary amino group determination

The trinitrobenzenesulfonic acid (TNBS) method (McKellar, 1981) was used to determine the primary amino group content of hydrolysates. A standard curve using L-leucine was used to determine μ mol. L-leucine/g.

Electrophoresis

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed using the method of Laemmli (1970) on 10% slab gels at a constant voltage of 105 v for 3.5 h. The protein bands were stained with Coomassie blue R-250. Molecular weight standards (myosin, β-galactosidase, phosphorylase B, serum albumin, ovalbumin, carbonic anhydrase, trypsin inhibitor and lysozyme) were applied on each gel for comparison and Rf values were plotted vs the log mol. wt. to establish a standard curve. Molecular weights of components in the
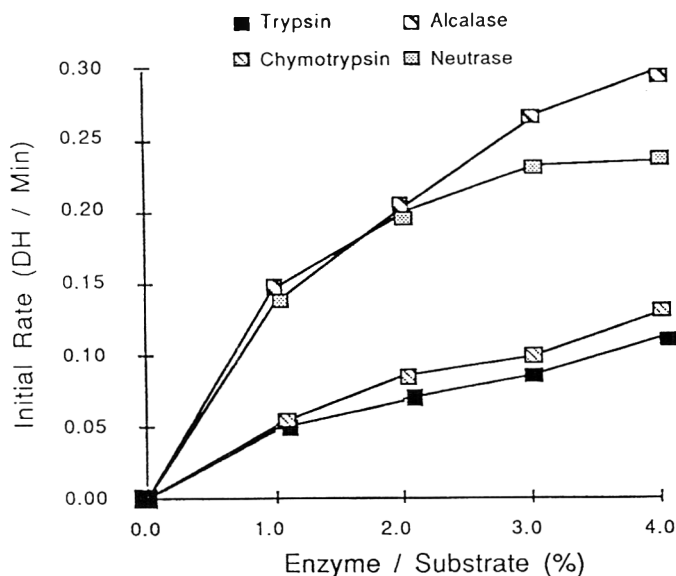
Fig. 2—Effect of enzyme substrate ratio on the initial reaction rate of hydrolysis of heat-denatured whey protein isolate by four enzymes.
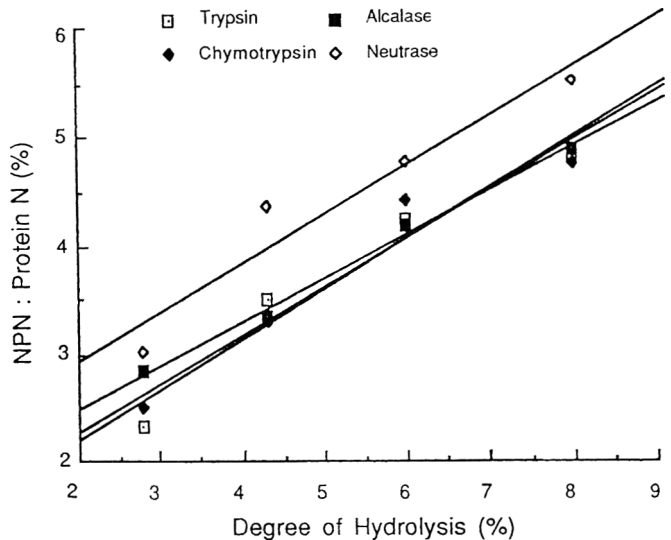

Fig. 3—Effect of degree of hydrolysis of heat-denatured whey protein isolate on the nonprotein nitrogen: protein nitrogen ratio for four enzymes.
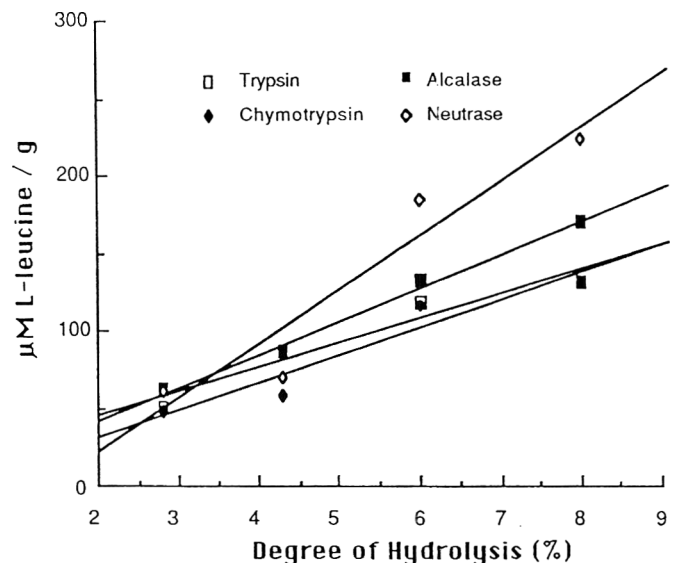
Table 1—Proximate composition of hydrolyzates prepared from heat-denatured whey protein isolate by trypsin, chymotrypsin, Alcalase and Neutrase

| | Moisture* (%) | | | |
	2.8% DH	4.3% DH	6.0% DH	8.0% DH
Trypsin	5.39 ± 0.03ᵃ	2.85 ± 0.22ᵇ	2.95 ± 0.06ᵇ	1.72 ± 0.13ᶜ
α-Chymotrypsin	4.57 ± 0.28ᵃ	5.24 ± 0.31ᵃ	3.99 ± 0.10ᵃ	4.28 ± 0.14ᵃ
Alcalase	2.45 ± 0.39ᵇ	2.08 ± 0.22ᵇ	1.32 ± 0.26ᶜ	2.61 ± 0.24ᵇ
Neutrase	0.30 ± 0.02ᶜ	2.22 ± 0.03ᵇ	2.57 ± 0.05ᵈ	4.28 ± 0.10ᵃ

| | Protein* (%) | | | |
	2.8% DH	4.3% DH	6.0% DH	8.0% DH
Trypsin	75.48 ± 0.45ᵃ	75.03 ± 0.89ᵃ	69.52 ± 0.21ᵃ	70.15 ± 0.07ᵃ
α-Chymotrypsin	71.46 ± 0.90ᵇ	71.01 ± 0.45ᵇ	70.05 ± 0.45ᵃ	69.94 ± 0.48ᵃ
Alcalase	69.97 ± 0.00ᶜ	70.57 ± 0.90ᵇ	64.63 ± 0.11ᵇ	66.16 ± 1.01ᵇ
Neutrase	71.90 ± 1.34ᵇ	72.35 ± 0.00ᵇ	67.76 ± 1.29ᵃ	62.13 ± 1.50ᶜ

| | Ash* (%) | | | |
	2.8% DH	4.3% DH	6.0% DH	8.0% DH
Trypsin	8.96 ± 0.04ᶜ	9.54 ± 0.04ᵈ	11.39 ± 0.05ᵈ	12.69 ± 0.03ᶜ
α-Chymotrypsin	10.54 ± 0.09ᵇ	10.48 ± 0.06ᶜ	11.66 ± 0.04ᶜ	11.46 ± 0.11ᵈ
Alcalase	16.46 ± 0.22ᵃ	14.10 ± 0.09ᵃ	16.17 ± 0.03ᵃ	17.03 ± 0.04ᵃ
Neutrase	10.30 ± 0.33ᵇ	13.01 ± 0.01ᵇ	15.04 ± 0.04ᵇ	16.45 ± 0.11ᵇ

* Values represent means of three replicates ± standard deviation. Means with similar superscripts do not differ significantly (p<0.05).

substrate (HDWPI) and the hydrolysis blanks were the same and were used to compare effects of hydrolysis.

Surface hydrophobicity

Surface hydrophobicity was measured using cis- parinaric acid by the procedure described by Kato and Nakai, (1980). Fluorescent intensities were recorded using a Turner Model 430 Spectrofluorometer at excitation wavelength 325 nm and emission wavelength 420 nm. Surface hydrophobicity (S_o) was defined as the initial slope of the curve of fluorescence intensity as a function of protein concentration.

Statistical analysis

Determination of compositional properties and surface hydrophobicity was accomplished by a completely randomized block design. All determinations were replicated 3 times except for mineral analysis which were replicated twice. Analysis of variance was performed on Statistical Analytical System (SAS Institute, Inc., 1985) programs. Duncan's Multiple Range Test was used to compare means.

Fig. 4—Effect of degree of hydrolysis on the primary amino groups (as µM of L-leucine) of digests of heat-denatured whey protein isolate.

RESULTS & DISCUSSION

Composition of WPC and HDWPI

The proximate composition of WPC varies over a wide range and was influenced by the source of whey and the fractionation method used. Normal protein contents of WPC's range from 35–50%. Our starting material, commercial WPC had 33.85 ± 0.39% protein, 0.22 ± 0.02% fat; 3.58 ± 0.05% moisture; 5.92 ± 0.02% ash; 56.44 ± 0.43% nitrogen free extract and NPN (as % protein nitrogen) content of 1.13 ± 0.05. After processing, the final product, HDWPI, had 76.60 ± 0.23% protein, 5.32 ± 0.06% fat; 4.28 ± 0.02% moisture; 3.80 ± 0.01% ash; 9.01 ± 0.08% NFE and 0.50 ± 0.02% NPN Conversion of WPC to HDWPI resulted in a significant increase in moisture, fat, and protein contents while there was a significant decrease in ash, NFE and NPN contents. Increases were attributable to concentration effects while decreases in ash, NPN and NFE were due to the diafiltration.

Conditions for inactivating enzymes

The optimum conditions of pH, temperature, and time for inactivation were determined for each enzyme. Trypsin was in-

Table 2—Approximate mol. wt. of hydrolysates of heat-denatured whey protein isolate from trypsin, chymotrypsin, Alcalase and Neutrase

Blank	2.8% DH				4.3% DH			
	Trypsin	Chymotrypsin	Alcalase	Neutrase	Trypsin	Chymotrypsin	Alcalase	Neutrase
121,300	123,100	121,900	—	—	—	—	—	—
111,100	—	—	—	—	107,900	113,400	—	—
95,500	95,600	102,800	100,400	100,400	98,800	102,800	97,900	100,400
—	86,700	88,800	84,600	88,800	82,600	86,700	84,600	88,800
—	78,600	—	—	—	—	—	—	—
65,700	61,600	60,100	60,100	61,600	61,600	—	60,100	61,600
55,600	58,700	—	—	53,200	—	—	—	—
52,000	53,200	—	—	—	—	—	—	53,200
47,200	—	43,800	46,000	47,100	—	43,800	46,000	—
—	30,400	27,500	28,200	29,600	—	—	—	—
26,200	24,400	—	—	25,000	28,200	27,500	28,200	28,900
—	—	—	23,800	23,200	—	—	23,800	24,400
21,900	—	22,600	22,600	—	22,600	22,600	22,600	23,200
—	16,900	15,300	15,000	15,700	16,500	15,700	16,100	15,700
—	16,100	—	—	—	16,100	15,000	15,000	15,300
13,100	—	14,200	13,900	—	13,900	—	13,600	—
12,700	—	—	—	—	—	—	—	—

Blank	6.0% DH				8.0% DH			
	Trypsin	Chymotrypsin	Alcalase	Neutrase	Trypsin	Chymotrypsin	Alcalase	Neutrase
121,300	—	—	—	—	—	—	—	—
111,100	—	—	—	—	—	—	—	—
95,500	100,400	—	—	—	—	—	—	—
—	—	—	—	—	—	—	—	—
65,700	66,300	—	—	—	—	—	—	—
55,600	—	—	—	—	—	—	—	—
52,000	—	—	—	—	—	—	—	—
47,200	—	—	—	—	—	—	—	—
—	32,600	31,900	31,100	31,900	33,500	31,900	32,700	31,900
26,200	26,900	26,900	26,200	26,900	26,900	26,900	26,900	26,900
—	24,400	24,400	25,500	24,400	24,400	23,800	24,400	24,400
21,900	—	—	—	—	—	—	—	—
—	—	—	—	—	—	18,100	—	—
—	—	—	—	—	—	17,700	17,700	—
—	—	—	—	—	—	16,900	17,300	16,900
—	16,500	16,900	16,500	16,500	—	16,500	16,500	16,100
—	16,100	16,100	16,100	16,100	—	—	15,000	—
—	—	—	15,300	—	—	—	—	—
13,100	—	—	—	14,200	—	—	—	—
12,700	—	—	—	—	—	12,900	—	—

activated at pH 10.5 at 65°C for 20 min; α-chymotrypsin at pH 4.5 at 68°C for 21 min; Alcalase at pH 3.0 at 56°C for 14.5 min while Neutrase had least activity after heating at pH 3.5 at 70°C for 21.5 min. Residual activity after inactivation was tested by incubation with HDWPI for 10 min. No base was consumed, confirming the complete inactivation of the enzymes. In a similar experiment active enzymes were incubated with the substrate at optimum temperature and after 10 min hydrolysis were subjected to inactivation treatments. After inactivation the assay system was again incubated at the optimum temperature to confirm that no further base was consumed.

For production of hydrolysates, after the desired DH was achieved, the activity of the enzymes was terminated. The HDWPI-enzyme systems were incubated for a further 10 min after inactivation and in all cases no more base was required confirming complete inactivation.

Effect of enzyme concentration at constant substrate

Hydrolysis was studied at four different E/S ratios of 1, 2, 3, and 4%, curves for the four enzymes (Fig. 1) were typical. Adler-Nissen (1977) reported similar hydrolysis curves for Alcalase on casein, maize isolate, soy isolate, wheat gluten, cottonseed protein concentrate and gelatin. The curves suggest a rapid reaction rate up to 20 min and a slower rate for the next 30 min. Possible reasons for decrease in reaction rates are loss of enzyme activity, substrate exhaustion or end-product inhibition. Loss of enzyme activity was not likely in the slow reaction zone. Substrate concentrations were in excess of the K_m values suggesting that substrate exhaustion was also unlikely.

Effect of substrate concentrations at constant enzyme

Initial reaction rates at three substrate concentrations (6, 8 or 10%) were not significantly different for any enzyme. When E/S ratio was increased from 2 to 4%, the reaction rate increased by a constant value irrespective of substrate concentration. The reaction rate increased by a factor of 2.0; 1.4; 1.5; and 1.3 for trypsin, α-chymotrypsin, Alcalase and Neutrase, respectively. These results also indicated that substrate concentration was sufficient to provide saturation conditions and was not a limiting factor. In most foods with a protein concentration of 4% or higher, substrate saturation is expected.

Since substrate exhaustion and enzyme inactivation were not probable reasons for decreasing reaction rates, there may be a competition between the substrate and peptides formed during hydrolysis. This type of inhibition was demonstrated by Adler-Nissen, (1986), though the chemical nature of such peptides has not been determined. Soy isolate was hydrolyzed to 10% DH with Alcalase and the insoluble hydrolyzates removed by centrifugation. The supernatent containing the soluble peptides and the substrate showed lower K_m values than for the unhydrolyzed isolate.

Other characteristics of hydrolysis of HDWPI

The maximum DH achieved with each enzyme increased with an increase in E/S ratio, and this was mainly due to an increase in reaction rate. A plot of initial reaction rate vs E/S for each enzyme confirmed this (Fig. 2). If formation of inhibitory peptides was the reason for slowing the hydrolysis, then the inhibitory effect was greater at low E/S ratio and decreased as E/S increased thus contributing to increased reaction rates. Also, the

Table 3—Surface hydrophobicity of hydrolysates from heat-denatured whey protein isolate by trypsin, chymotrypsin, Alcalase and Neutrase

	Surface hydrophobicity*			
	2.8% DH	4.3% DH	6.0% DH	8.0% DH
Trypsin	238.68 ± 3.04[b]	308.28 ± 1.22[b]	251.04 ± 1.58[c]	184.49 ± 1.05[d]
α-Chymotrypsin	169.29 ± 1.57[c]	226.46 ± 0.04[c]	284.07 ± 3.15[b]	293.76 ± 1.86[a]
Alcalase	304.27 ± 3.08[a]	202.35 ± 1.01[d]	317.98 ± 1.73[a]	252.88 ± 0.16[c]
Neutrase	162.21 ± 1.41[d]	325.28 ± 2.65[a]	245.00 ± 3.62[d]	287.63 ± 2.10[b]

* Values represent means of three replicates ± standard deviation. Means within columns with similar superscripts do not differ significantly ($p < 0.05$).

rate of splitting certain peptide bonds appeared to increase proportionally with increase in E/S (Alder-Nissen, 1982). The relationship between initial reaction rate and E/S (Fig. 2) was not linear. The deviation from linearity can be attributed to the difference in solubilities between HDWPI and its hydrolysates. With insoluble hydrolysates, adsorption of substrate on the enzyme becomes rate limiting since only enzyme adsorbed to the insoluble substrate participates in the reaction (McLaren and Parker, 1970). The initial reaction rate (v) is a power function of E/S or $v = k (E/S)^n$ where $n = 1$ and k is a rate constant (McLaren, 1963). Using this equation the relationships for the four proteases were as follows: trypsin, $v = 0.046(E/S)^{0.59}$; α-chymotrypsin, $v = 0.051(E/S)^{0.66}$; Alcalase $v = 0.137(E/S)^{0.58}$; Neutrase $v = 0.151(E/S)^{0.36}$. These results confirmed the importance of adsorption in hydrolysis as n for all reactions was <1. The values of n for trypsin and Alcalase corresponded to the values obtained for hydrolysis of soy isolate at pH 4.5 with *Aspergillus niger* protease Sumzyme AP (Adler-Nissen, 1986). The order of decrease in reaction rate at each E/S level was Neutrase, Alcalase, α-chymotrypsin and trypsin. There were no significant differences in reaction rates of Alcalase and Neutrase at E/S levels of 1 and 2%, respectively. The rate constants for α-chymotrypsin and trypsin were smaller than those of Alcalase and Neutrase at each E/S level. This suggested that the broader the specificity of the enzyme, the higher the initial rates. Specificity in all enzymes was confined to endopeptidase activity as the enzymes tested negative for either carboxypeptidase or aminopeptidase activities.

Proximate composition of hydrolysates

The moisture, protein and ash content of the freeze-dried hydrolysates at four DH levels were compared (Table 1). Considerable differences were observed in moisture though no trend or pattern was noticed. The differences in moisture could arise from varying efficiency of freeze drying or storage conditions. Ash content of the hydrolysates increased with increasing DH for each enzyme. Alcalase hydrolysates had higher ash content than any other hydrolysates. Increase in ash content corresponded to the increase in base consumption with increasing DH. The adjustment of pH during enzyme inactivation or neutralization before freeze drying could also contribute to increased ash content.

Trypsin hydrolysates produced at 2.8 and 4.3% DH were not different from unhydrolyzed HDWPI in protein content but were higher than other hydrolysates. Protein contents tend to decrease with increase in DH. The differences in protein content of the hydrolysates at various DH were due to the corresponding increase in NPN. Neutrase hydrolysates had the highest NPN values at all DH levels. A linear relationship between NPN and extent of hydrolysis was observed with each enzyme (Fig. 3).

Conversion of WPC to HDWPI resulted in an increase in Ca (483.05 to 671.70 mg/100g), Mg (50.50 to 59.60 mg/100g), and Na (385.00 to 390.45 mg/100g) while the K (771.15 to 306.00 mg/100g) and P (568.85 to 391.35 mg/100g) contents decreased. The balance between free soluble and colloidal complexes of minerals determines the quantity of minerals remaining after ultrafiltration. Only the free soluble forms pass through the membrane while colloidal complexes would be retained. The balance between free soluble and colloidal forms of minerals is influenced by pH. Lowering the pH increases the free soluble form and facilitates removal by ultrafiltration (Hiddink et al., 1978). In our study ultrafiltration was performed at pH of 6.3 resulting in an increased retention of Ca, Na, and Mg.

The Ca, Mg, K, and P content of the hydrolysates did not differ from the corresponding unhydrolyzed control. Sodium contents increased with increase in DH content for all four enzymes. This was because more base (NaOH) was needed to neutralize the protons liberated during hydrolysis. This trend in Na content paralleled the trend in ash content discussed earlier.

Primary amino groups

Primary amino groups increased through the course of hydrolysis and the increase was linear with respect to DH (Fig. 4). The number of primary amino groups generated depends on the size of peptides produced. Shorter peptides generate higher primary amino nitrogen than do longer peptides. Enzyme specificity is a key factor controlling the generation of primary amino groups. Alcalase and Neutrase have relatively broad specificities and would generate hydrolysates with more primary amino groups than produced by trypsin or α-chymotrypsin. Monti and Jost (1978) reported that the neutral protease from *Bacillus subtilis* produced highest α amino groups compared to trypsin and papain during the solubilization of lactalbumin. The starting material, WPC had 3.26 μ mole leucine/g and HDWPI contained 4.46 μmole leucine/g. These values were not different (p > 0.05) indicating that no proteolysis occurred during manufacture of HDWPI.

Electrophoresis

The approximate molecular weights of hydrolysates at different degrees of hydrolysis for the four enzymes were compared (Table 2). The data revealed differences in proteolytic patterns of the different enzymes. At 2.8% DH for all enzymes, the 12,700 mol wt component was absent, indicating a preferential and early hydrolysis of this low mol. wt. component. Trypsin and chymotrypsin did not breakdown the 121,300 component which was, however, hydrolyzed by both Alcalase and Neutrase. At 4.3% DH, however, this component had been hydrolyzed by both trypsin and chymotrypsin. The component with mol. wt. 111,100 was hydrolyzed by all four enzymes at 2.8% DH and did not show up on the gel.

After hydrolysis had progressed to 8.0% DH, no component with mol. wt. >35,000 was observed for any of the enzymes. Trypsin hydrolysate showed only 3 distinct bands while chymotrypsin, Alcalase and Neutrase showed 8, 7 and 5 bands, respectively. The molecular weights of hydrolysates were distributed over a much wider range at 2.8% and 4.3% DH than at 6.0 or 8.0% DH. This suggested the formation of fragments of intermediate molecular weights during hydrolysis and their subsequent breakdown with progress of hydrolysis. Proteolytic behavior, where the proteins are fragmented to intermediates before further hydrolysis to smaller peptides, has been described as 'zipper mechanism' (Adler-Nissen, 1976). The substrate in this case was heat-denatured and aggregated and had components of a wide range of molecular weights. New intermediates, which did not correspond to any of the initial fractions were created during the course of hydrolysis (at 2.8% and 4.3 DH)

and were subsequently hydrolyzed to smaller fragments (at 6.0 and 8.0% DH).

Surface hydrophobicity (S_o)

Surface hydrophobicity is a measure of hydrophobic residues exposed at the protein or peptide surface and is a sensitive indicator of conformational changes (Kato and Nakai, 1980). Such an indicator may be useful in relating the structure of proteins to their functionalities. The S_o of whey protein before heat denaturation was 389.9 which decreased to 264.0 after thermal denaturation and aggregation. Hydrophobic interactions, leading to fewer exposed hydrophobic residues and sulfhydryl interchange reactions could be responsible for the decrease in S_o.

When HDWPI was hydrolyzed, there was a significant increase in S_o at each DH level for each enzyme (Table 3). Hydrolysis of peptide bonds could be expected to uncover hydrophobic groups previously involved in aggregations. We therefore, anticipated that S_o would increase linearly with DH. This was observed for hydrolysates of α-chymotrypsin but not with the other three enzymes, indicating that α-chymotrypsin hydrolyzed peptide bonds involving the hydrophobic amino acids, phenylalanine, tyrosine, tryptophan, and leucine. Release of these residues may increase S_o values of the hydrolysates.

In other hydrolysates the lack of a linear increase in S_o with increase in DH was consistent with the lack of linear increase in number of bands in DISC-PAGE. Self association or formation of new hydrophobic interaction with peptide fragments may be additional factors involved in this. In trypsin hydrolysates, S_o increased as DH increased from 2.8% to 4.3% and then progressively decreased as DH increased to 8.0%. Trypsin was inactivated at pH 10.5 for 21 min at 65°C. The pK of thiol group is ≈8.0 (Li-Chan, 1983) and therefore at > pH 8.0 sulfhydryl reactions with intermolecular hydrophobic interactions were favored and may have caused a decrease in exposed hydrophobic groups leading to a decreased S_o value. Neutrase hydrolysates exhibited a trend similar to trypsin hydrolysates whereas Alcalase hydrolyzate showed a bimodal increase in S_o. At 2.8% and 6.0% DH, the S_o values for Alcalase hydrolyzate were higher than for the other hydrolysates. This could be partially explained by the association of the peptides through hydrophobic interactions.

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Ms received 11/14/94 revised 4/7/95; accepted 4/13/95.

HIGHLY SENSITIVE UREA DETECTION METHOD. . .From page 1099

commercial sherry, and in red and white wines. The method shows promise as a general method for detection of trace amounts of urea in beverages.

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Ms received 1/27/95; revised 3/6/95; accepted 3/31/95.

Screening of Proteolytic Enzymes to Enhance Foaming of Whey Protein Isolates

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ABSTRACT

Whey protein isolate (WPI) was modified to enhance foaming characteristics by controlled hydrolysis using proteolytic enzymes. Alcalase, acid fungal protease, chymotrypsin, pepsin and trypsin were used to hydrolyze 5% rehydrated WPI. Degree of hydrolysis was estimated by freezing point depression and terminated at 2.5 to 3% by heating or pH adjustment. Controls were treated under similar conditions but without enzymes. Hydrolysates were separated into permeate and retentate by ultrafiltration and concentrated by reverse osmosis before freeze drying. Foam capacity, stability and surface tension of hydrolysates were measured. Permeate from Alcalase exhibited the best foaming characteristics, comparable to egg white.

Key Words: whey protein isolate, proteolysis, foaming properties, alcalase

INTRODUCTION

WHEY PROTEINS are used as functional ingredients in food systems. Whey protein functionality includes solubility, whippability, viscosity, gelation, water binding, foaming stability, and emulsifying capacity. Products like dry whey, whey protein concentrates, whey protein isolates, partial whey products and specialized fractions are used as ingredients in beverages, baked goods, confectionery, pasta, meat, dairy products and analogs (Kilara, 1994).

Egg white is the preferred foaming agent in many food systems because it whips easily and rapidly generates large volumes of stable foam. However, egg white is more costly than whey. Attempts to improve whey protein functionality have included chemical and enzymatic modifications. Heating whey, causing partial denaturation of the protein, marginally improved foaming (Schmidt and McNeill, 1993). Limited proteolysis improved whey protein foaming but increased foam capacity was accompanied by a lowering of foam stability (Kuehler and Stine, 1974), while reduction of disulfide bridges between subunits increased foam capacity and stability (German et al., 1985).

Five enzymes: trypsin, pepsin, acid fungal protease (AFP), chymotrypsin and Alcalase, were used to hydrolyze WPI and the foaming characteristics of the peptide fractions obtained by ultrafiltration were compared. Our objective was to improve the foaming characteristics of WPI and make it comparable to egg white.

MATERIALS & METHODS

Proteolysis

Bi-Pro whey protein isolate (WPI) was obtained from Davisco International Inc., (Le Sueur, MN); acid fungal protease from Gist-brocade (Charlotte, NC); Alcalase 0.6 from Novo Nordisk Bioindustrials, Inc. (Danbury, CT); and trypsin, α-chymotrypsin and pepsin from Sigma (St. Louis, MO). The WPI, containing 95% protein, was reconstituted at 5% (w/v) in tap water. The temperature and pH were adjusted to the optimum for each enzyme at the start of hydrolysis (Table 1), except for Alcalase, which was incubated at 30 °C instead of its optimum of 50 °C. Lowering the temperature slowed the rate of reaction, and allowed

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better control of the degree of hydrolysis. Enzyme/substrate ratios were adjusted to control the rate of reaction and achieve similar degrees of hydrolysis in comparable times. Controls were prepared simultaneously for each enzyme, under identical conditions but without added enzyme. Once hydrolysis was initiated, the pH was not adjusted.

Each enzyme was incubated until a 2.5 to 3% degree of hydrolysis (DH) was achieved. Degree of hydrolysis was determined by the freezing point depression method described by Alder-Nissen (1986). A milk cryoscope model 4L II (Advanced Instruments, Inc., Needham Heights, MA) was used to measure the freezing points of 2 mL aliquots taken at different times during hydrolysis. A 21 m°C depression indicated a 2.7% DH as determined by the following equation:

$$\% \text{ DH} = \frac{\Delta C}{S \%(f_{osm})} \times \frac{1}{\omega} \times \frac{1}{h_{tot}} \times 100$$

where ΔC = depression of freezing point in milli-osmol; S% (f_{osm}) = (g protein)/(1000 mL H$_2$O); $1/\omega$ = calibration factor for the osmometer = 1.04; and h_{tot} = total number of peptide bonds in the protein substrate (WPI = 8.8 meqv/g protein; Alder-Nissen, 1986).

Proteolysis was terminated by either heating to 85 °C for 15 min or by adjusting the pH to 7.0. Alcalase, chymotrypsin and trypsin were inactivated by heating, and pepsin and AFP were inactivated by neutralizing the pH.

After termination of enzyme action the hydrolysates were separated into permeate and retentate by ultrafiltration using a Millipore Pellicon cassette ultrafiltration unit (Millipore Corporation, Bedford MA) with a 10,000 MW cutoff membrane. The retentate was recirculated until the permeate was clear and then diafiltered with two volumes of water to ensure complete separation of low molecular weight peptides. Each enzyme treatment yielded four fractions: hydrolysate permeate (HP), hydrolysate retentate (HR), control permeate (CP) and control retentate (CR). In the treatment with Alcalase a curd was formed and had to be pre-filtered with a cheese cloth before ultrafiltration.

Each fraction generated by ultrafiltration was concentrated by reverse osmosis using a RO-system Lab Module DDS Filtration Type 20, HR membrane (Niro Hudson, Inc., Hudson WI). The concentrated samples were frozen, lyophilized (VirTis Inc., Gardiner NY) and stored dry.

Foaming

Foam capacity and foam stability was measured for each fraction. The quantity of permeate fraction from the controls was insufficient to perform these tests. The foam capacity and foam stability tests were as outlined in Phillips et al. (1987, 1990). Freshly prepared sample (75 mL 5% solution) adjusted to 7.0 pH was used for each run of both capacity and stability tests. A Sunbeam Mixmaster PowerPlus mixer was used for whipping. The foam formation procedure required measurement of the weight of 100 mL of foam at 5, 10 and 15 min of whipping at 78 rpm. Overrun was calculated from the following equation:

$$\% \text{ Overrun} = \frac{(\text{wt 100 mL protein}) - (\text{wt 100 mL foam})}{\text{wt 100 mL foam}} \times 100$$

Foam stability was measured by whipping the solution for 10 min and measuring the time in sec required for half the volume of foam to drain through an uncovered hole into a tared weighing dish. Tests were performed in triplicate for each fraction, and results averaged.

Surface tension

Surface tension was measured by drop volume technique using a Lauda Drop Volume Tensiometer TVT1 (Brinkmann Instruments, Inc., Westbury, NY), which comprised of a measuring unit and control unit connected to a computer (IBM-PS2, model 50Z) and a printer. Surface

Table 1—Conditions for enzyme incubation and inactivation, and final degree of hydrolysis (DH)

Enzyme	Incubation			Inactivation			
	Enzyme/Substrate Ratio (%)	pH	TEMP (oC)	TEMP (oC)	TIME (min · sec)	pH	DH (%)
Alcalase	0.8	8.5	30	85	15.00	7.0	3.1
Chymotrypsin	0.5	7.8	25	85	15.00	7.0	3.1
Trypsin	0.5	7.6	25	85	15.00	7.0	2.8
Pepsin	0.5	2.0	37	37	9.30	7.0	2.7
Acid fungal protease	1.0	5.0	30	30	10.50	7.0	3.1

Table 2—Foaming characteristics and surface tension of hydrolysates from five enzymes compared with heated whey, unmodified whey and egg white

Enzyme	Fraction	Overrun (%)	Stability (sec)	Surface tention (mN/m)
Heated whey		756 ± 14[bcd]	2229 ± 57[g]	69.14 ± 0.64[klm]
Whey		603 ± 59[b]	1084 ± 194[c]	70.07 ± 0.65[m]
Egg white		833 ± 57[bcd]	1742 ± 67[e]	69.04 ± 0.13[kl]
Alcalase	Control retentate	0*[a]	0**[a]	68.62 ± 0.64[k]
	Control permeate	ND	ND	60.71 ± 0.57[g]
	Hydrolysate retentate	2018 ± 70[f]	1961 ± 108[f]	58.99 ± 0.55[de]
	Hydrolysate permeate	3169 ± 212[g]	2540 ± 187[h]	56.55 ± 0.53[b]
Chymotrypsin	Control retentate	0*[a]	0**[a]	69.91 ± 0.60[lm]
	Control permeate	ND***	ND	62.27 ± 0.58[h]
	Hydrolysate retentate	1571 ± 386[e]	1393 ± 165[d]	59.30 ± 0.55[ef]
	Hydrolysate permeate	2049 ± 224[f]	1345 ± 198[d]	57.47 ± 0.54[bc]
Trypsin	Control retentate	0*[a]	0**[a]	69.96 ± 0.65[lm]
	Control permeate	ND	ND	64.15 ± 0.49[l]
	Hydrolysate retentate	1169 ± 117[de]	0**[a]	60.00 ± 0.56[fg]
	Hydrolysate permeate	966 ± 31[cd]	1737 ± 94[e]	57.94 ± 0.54[c]
Pepsin	Control retentate	656 ± 11[bc]	1758 ± 41[e]	67.02 ± 0.63[j]
	Control permeate	ND	ND	64.90 ± 0.61[i]
	Hydrolysate retentate	744 ± 45[bcd]	1034 ± 25[c]	58.24 ± 0.47[cd]
	Hydrolysate permeate	967 ± 63[cd]	485 ± 78[b]	54.98 ± 0.51[a]
Acid fungal protease	Control retentate	776 ± 18[bcd]	1988 ± 43[f]	64.34 ± 0.60[i]
	Control permeate	ND	ND	68.46 ± 0.64[k]
	Hydrolysate retentate	0*[a]	0**[a]	66.31 ± 0.62[j]
	Hydrolysate permeate	2101 ± 1107[f]	0**[a]	69.82 ± 0.65[lm]

[a-m] Figures in columns with unlike superscripts differ (p < .05).

* Insufficient foam was produced.

** Foam had a stability less than 1 min.

*** ND = Foam experiments not done due to insufficient material.

tension was measured, by quasistatic method (Patel, 1994). Drops of the protein solution were formed at the tip of a capillary (1.3 mm radius) attached to a gas-tight precision syringe (2.5 mL, Hamilton Series 1000). The liquid in the syringe was transferred to the tip of the capillary in small increments (10^{-2} μL) and the number of increments required to form a drop was counted to determine the drop volume. The detachment of the drop from the capillary was detected optically. The computer software supplied with the equipment calculated the surface tension of the solution from the drop volume. In the quasistatic method, a drop of protein solution of sub-critical volume was allowed to form quickly at the tip of the capillary. Protein adsorption at the surface increased with time, causing a simultaneous decrease in interfacial tension and the drop detached when its volume became critical. This procedure measured the change in surface tension with effective age of the interface.

Samples (0.1%) were prepared in distilled water, and the pH adjusted with either 0.5N HCl or 0.25N NaOH. Any protein particles that might sediment during the surface tension measurement were removed by centrifugation for 15 min at 3000 × g. All protein solutions were prepared fresh each day.

RESULTS & DISCUSSION

A 2.5 to 3% DH was intended for each enzyme, in order to maintain uniformity and enable comparison between enzymes. Proteolysis, however, could not be terminated instantly, and the time required for termination allowed additional proteolysis beyond 3% DH. When heat was used to inactivate the enzyme, coming-up time had to be allowed, and the intermediary rise in temperature would accelerate reaction rate before enzyme inactivation. A freezing point depression of 21 m°C was calculated to correspond to 2.7% DH. Termination of the reaction was initiated when the freezing point depression approached, but was still less than, 21 m°C, allowing some inevitable additional hy-

drolysis to occur before complete termination. Even with this allowance, the final DH could not be controlled within ±2.5%. On average, an additional 0.6–0.8% DH would occur during a heat termination of the reaction.

Foam capacity was measured by the % overrun in a 5% solution after whipping (Table 2). Foaming tests could not be conducted for permeates from the controls due to insufficient material. The retentates from the control represent the foaming characteristics before enzyme modification in each case and had low foam capacities. The controls were heated or neutralized differently for each enzyme to parallel the enzyme treatment and this could account for differences in foam capacities. Retentates from controls for chymotrypsin and trypsin produced very little and unstable foam even after 15 min whipping and could not be measured. Unmodified, untreated WPI showed an overrun of 603% and the controls for pepsin (656%) and AFP (776%) were close to this value. In both cases, pH adjustment was used and not heat treatment as in the other three controls.

Enzymatic hydrolysis altered the overrun of hydrolysates in all cases as compared to controls. Controls were not hydrolysed, so there was very little material with molecular weight less than 10,000 in the permeate. The permeates of the hydrolyzates showed better foam capacities than retentates for all enzymes except trypsin. The permeate fraction contained the lower molecular weight range peptides. Enhanced foaming capacities as a result of limited hydrolysis has been attributed to the increase in polypeptide content allowing more incorporation of air (Kuehler and Stine, 1974). Good foaming is a function of rapid migration of peptides to the air-water interface and their rapid unfolding at the surface (Halling, 1981). The reason for the exceptional behavior of trypsin, where retentate (larger mol. wt.

range peptides) foamed better than permeate, is not clear. The foam capacities increased to varying extents but the permeate from Alcalase showed the best foaming with an overrun of 3169%.

Foam capacities of unmodified whey, partially heat denatured whey (55°C for 30 min) and egg white were measured under similar conditions for comparison (Table 2). The permeate from Alcalase treatment performed better than the others with regard to foam capacity.

Foam stability observations were different from foam capacity. Controlled proteolysis did not necessarily improve foam stability. The fractions generated by chymotrypsin, trypsin and Alcalase showed better foam stability than the control. Pepsin did not alter the foam capacity but seemed to decrease foam stability of the fractions. Hydrolysis by AFP produced fractions which could generate high volume (2101% overrun), but transient foam with no stability.

Foams disintegrate due to capillary drainage of the lamellae and due to collapse of bubbles. Resistance to these two factors determines foam stability and is dependent on the rheological and adhesive properties of interfacial films (Halling, 1981). In our study, foam stability was measured as a function of draining (Phillips et al., 1990). It has been suggested that retention of secondary and tertiary structure at the interface aids foam stability (Graham and Phillips, 1979). Proteins with a high molecular weight have been reported to show greater film strength. Even partial hydrolysis reportedly may lower foam stability (Adler-Nissen, 1986). German et al. (1985) showed that a reduction of disulfide linkages between subunits sharply increased foam capacity and stability. They reasoned that their treatment allowed rapid and complete exposure of the hydrophobic regions of the subunits at the expanding surface and also promoted inter-subunit associations into an aggregated film network. Functional properties of the entire hydrolyzed material were studied and no attempt was made to fractionate or separate peptides from un-hydrolyzed portions. In our study lower molecular weight peptides (<10,000) were separated and concentrated as the permeate. Possibly some of the larger molecular weight peptides could have an anti-foaming influence, resulting in lower foam capacity or foam stability.

Surface tension results (Table 2) indicated the presence of surface-active peptides. The capacity to form a protein film is related to the ability to decrease surface tension. Foam stability is influenced by the nature of the film, which in turn, is dependent on the extent of protein-protein interaction within the film matrix (German and Phillips, 1994). However, there seemed to be no relationship between foam capacity or foam stability and surface tension reduction. There were no apparent relationships between extent of surface tension depression and foaming capacity and stability. A sample with high surface activity would not necessarily foam well or form a stable foam. The surface tension values for unmodified WPI were similar to those reported by Tornberg (1978) for whey protein concentrates.

Limited hydrolysis can improve whey protein characteristics, depending on the type of enzyme and extent of hydrolysis. Kuehler and Stine (1974) reported that while limited hydrolysis by prolase, pepsin and pronase improved initial foam capacity, it decreased foam stability. The results of pepsin hydrolysis in our study confirmed their report.

The nature of peptides and ease with which they migrate to the surface and rearrange or denature determines foam capacity. Residual tertiary structure and interaction with other subunits or peptides influences foam stability (German and Phillips, 1994). Controlled proteolysis of whey proteins with different proteolytic enzymes generates a wide variety of peptides with a wide range of functional properties. Our study showed that limited hydrolysis with Alcalase produced a fraction that had excellent foaming characteristics. This fraction showed better foam capacity and foam stability than unmodified WPI, heat denatured WPI or egg white (Table 2). The method we used could consistently produce a fraction from WPI with better foaming properties than egg white. Therefore, this fraction shows promise as an egg white replacer in food systems, such as confections, baked goods and whipped toppings.

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Ms received 8/1/94; revised 5/05/95; accepted 5/13/95.

Antioxidant Activity of Fungus *Suillus bovinus*
(L: Fr.) O. Kuntze

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ABSTRACT

Two major antioxidative compounds were isolated from wild mushrooms, *Suillus bovinus* (*L: Fr.) O. Kuntze*, and studied for antioxidative activity by comparison with other antioxidants, BHA and tocopherol. One compound was an orange pigment identified as variegatic acid (3,3',4,4'-tetrahydroxy puluvinic acid) and the other was an orange-red pigment, possibly diboviquinone-4,4. The variegatic acid had strong antioxidative activity in an emulsion system as shown by peroxide value (POV) of linoleic acid and in an oil system as shown by the POV of methyl linoleate and weight gain of soybean oil. The second compound was active only in the emulsion system.

Key Words: fungus, *Suillus bovinus*, antioxidant, tocopherol, diboviquinone

INTRODUCTION

THE LIMITATIONS of synthetic antioxidants such as BHA, BHT, TBHQ and others have been shown in reviews of toxicity (Branen, 1975), cancer (Ito et al., 1983) and promutagenic activity. This has resulted in increased demands for natural antioxidants. Much attention has been focused on antioxidant mechanisms from natural sources.

We screened species of edible plants (Kasuga et al., 1988) and mushrooms (Kasuga et al., 1993) for antioxidative activity in an effort to discover natural antioxidants. In a previous report (Kasuga et al., 1993), several extracts, particularly those from the mushroom, *Suillus bovinus (L.: Fr.) O. Kuntze*, were shown to have strong antioxidative activity.

Suillus bovinus, an edible mushroom, of the *Suillus* species of *Boletaceae*, grows in forests of pines from summer to autumn in Japan. Its pileus is 5–11 cm in diameter, and its stem, 3–6 cm in length. The surface is sticky and the collar is yellowish brown. After cooking, the collar turns red violet.

Our objective was to isolate antioxidative compounds from *Suillus bovinus* and compare activities with other antioxidants, BHA and tocopherol.

MATERIALS & METHODS

Reagents

Methyl linoleate was purchased from Tokyo Kasei Co. (Tokyo, Japan) and linoleic acid and soybean oil, from Wako Chemical Ind. (Osaka, Japan). All other chemicals were of reagent grade.

Preparation of samples

Suillus bovinus was obtained from Nagano prefecture in Japan from 1984 to 1992. After harvesting, all mushrooms were thoroughly washed and rinsed several times with distilled water and frozen. After being lyophilized, sporophores were powdered.

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Extraction and purification of antioxidative compounds

Compound I. Lyophilized powder, corresponding to 7.5 kg fresh weight, was extracted with 3L of 70% ethanol at 80°C and filtered. The residue was further extracted two times, and all extracts were combined and evaporated. The concentrates were extracted with 1L hexane, ethyl acetate and butanol successively. An aliquot of butanol phase was loaded onto a silica gel column (Wakogel C-200; Wako Chemical Ind. (Osaka, Japan), 35ø × 450 mm) and eluted successively with 1L volumes of chloroform - methanol - acetic acid - water(70:15:12:3, 60:20:15:5, 40: 30:15:15, 20:50:15:15). The fraction eluted with the solvent of 60:20: 15:5 ratio was concentrated and passed through a gel-filtration column (Toyopearl HW-40F; Tosoh Co. Tokyo, Japan, 26ø × 90 mm). The column was eluted with 1% pyridine in 80% methanol. The eluate was further purified by application onto a cation column (Dowex 50 × 4 20ø × 100 mm) with 50% methanol. The eluate was concentrated and crystallized from water. Compound I was obtained by recrystallization from water.

Compound II. Lyophilized powder, corresponding to 23 kg fresh weight, was immersed in 35L 70% ethanol at room temperature over night and filtered. The residue was extracted with 15L 70% ethanol, and both extracts were combined. An excess of 10% aqueous lead acetate was added to the extracts and the resulting dark olive-green precipitates were filtered. The precipitate was washed with water and methanol. The lead salt was suspended in 2L methanol and 2L 5% methanolic hydrogen chloride added and filtered. The red filtrate was evaporated until hydrochloric acid had been removed. Compound II was obtained by recrystallization of the resulting red precipitate from acetic acid.

Test for antioxidant activity

The antioxidant effectiveness of the isolated compounds and known standards was ascertained by determining (1) peroxide value (POV) of methyl linoleate in oil phase, (2) POV of linoleic acid in the emulsion phase, and (3) weight gain of soybean oil.

For the first determination, 50μL ethanol solutions containing 100 μg, 10 μg and 1 μg of compound I and II respectively, were added to 100 μL methyl linoleate n a tube (1.5ø × 7.5 mm). By the method of Nose and Fujino (1982), the reaction mixtures were incubated at 50°C in a dry block bath. After 24 hr, POV was measured by the modified method of Lea (JOCS, 1990). Antioxidant activity was expressed as the ratio of POV of test solution to control.

The second method for estimating antioxidant effectiveness was to determine POV of linoleic acid in the emulsion phase. The compounds dissolved in 1 mL ethanol, 8 mL of 0.05 M linoleic acid in ethanol, 8 mL of 0.1M phosphate buffer pH 7.0 were mixed and volume was adjusted to 20 mL with distilled water. The concentrations of test samples were 5 mg or 0.05 mg/100 mL of reaction mixtures. The mixtures were incubated at 40°C in a dark room and at specified intervals, 0.1 mL aliquots were removed and POV was determined colorimetrically by the ferric thiocyanate method (Mitsuda et al., 1966).

As the third method for estimating antioxidant effectiveness, the weighing method for measuring the induction period of compounds (JOCS, 1990) was used. To 45 mm i.d. glass petri dishes, 1.000 ± 0.003g soybean oil were added. Accurately measured amounts of the antioxidants, 0.1, 0.2, 0.5, and 1 mL of a solution of each compound containing 1 mg/mL in ethanol, corresponding to 0.01%, 0.02%, 0.05%, and 0.1% to the oil respectively, were added. The dishes were placed in a constant temperature oven at 60°C without covers. After 20 min the solvents were removed and contents of the dishes were rotated to insure uniform distribution of antioxidants in the oil. They were then covered. At daily intervals, the dishes were removed from the oven, allowed to cool in a desiccator for 30 min, weighed, and placed again in the oven. Antioxidant effectiveness was expressed as the induction period required for 5% weight gain of soybean oil.
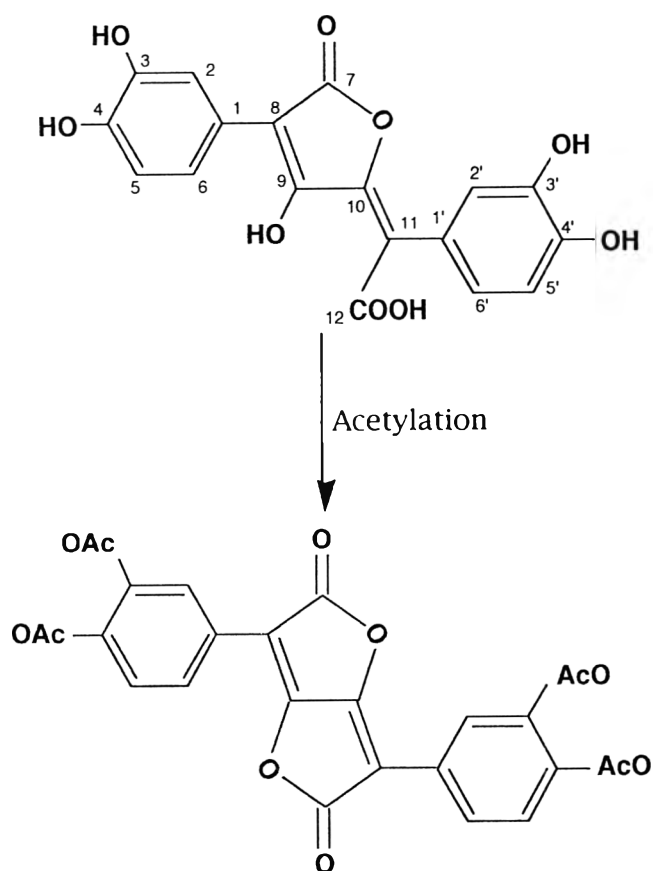
Fig. 1—Structures of variegatic acid and its acetylate.

Acetylation
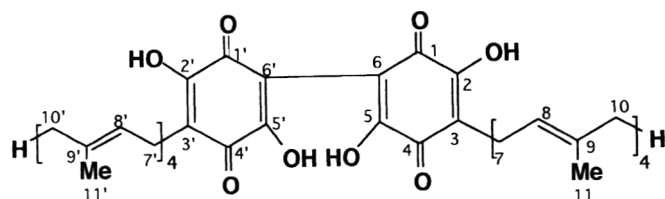
Fig. 2—Structure of diboviquinone.

Instruments for structural determination

The physicochemical properties of the compounds were measured using the following instruments. YANAKO MT-2 (elemental analysis) JASCO A-202 (IR) JEOL JNM-EX 270 (FT-NMR) HITACHI M-80 (MS).

RESULTS & DISCUSSION

Identification of compound I

We previously reported *Suillus bovinus* produced various antioxidative compounds which dissolved in polar solvents (Kasuga et al., 1993). As a preliminary test, *Suillus bovinus* was extracted with chloroform, ethanol and 70% ethanol. The chloroform and ethanol extracts were fractionated by column chromatography but antioxidative activity gradually became weak (data not shown), possibly due to the presence of minor antioxidative compounds. In this experiment, 70% ethanol extraction was used in which antioxidative compounds were not dispersed.

From the lyophilized powder of 7.5 kg fresh *Suillus bovinus*, 450 mg of compound I were crystallized from water and obtained as an orange powder. Various fractionations were carried out while measuring antioxidative activity. The melting point was 240°C (decomp.). From elemental analysis, the molecular formula of compound I was $C_{18}H_{12}O_9 \cdot H_2O$ (Found; C,54.6;

Table 1—Antioxidant activity of isolated compounds in an oil system[a]

	100 µg	10 µg	1 ug
BHA	+ +	+ +	-
α-Tocopherol	+ +	-	-
compound I	+ +	+ +	-
compound II	+	-	-

[a] + + POVs/POVc<0.2; + 0.2≤POVs/POVc<0.5; - 0.5≤POVs/POVc; POVs = Peroxide value with the sample; POVc = Peroxide value of control. The mixture of 50 µL methyl linoleate and 100 µL EtOH containing test samples were incubated at 50°C. POV was measured by the modified method of Lea (JOCS, 1990).

H,3.6; O,41.8 Calculated C,55.7; H,3.6; O,41.0). The spectroscopic data were as follows. IR ν_{max} (KBr disk) cm^{-1}; 3390 (-OH), 1762(-COOH), 1692(-CO), 1625, 1598, 1535, 1382, 1293, 1015, 910, ^{1}H-NMR δ(CD$_3$OD); 7.63 (1H,dd), 7.49 (1H,dd), 6.81~6.77 (3H,m), 6.67 (1H,dd), ^{13}C-NMR δ(CD$_3$OD); 105.7 (C-8 or C-11), 116.3 (C-2'), 116.5 (C-2), 116.7 (C-5), 118.9 (C-11 or C-8), 119.1 (C-5'), 121.9 (C-6), 123.1 (C-1), 123.9 (C-6'), 126.5 (C-1'), 146.2 (C-4'), 146.6 (C-3'), 147.3 (C-4), 147.4 (C-3), 154.8 (C-9 or C-10), 161.3 (C-10 or C-9), 169.3 (C-7), 175.2 (C-12), EI-MS m/z; 372 (m⁺), 354, 298, 177, 149. The data of ^{1}H-NMR were in good agreement with published data (Wolfgang et al., 1974). When this compound was acetylated, the analysis data were as follows. IR ν_{max} (KBr disk) cm^{-1}; 1828, 1775, 1660, 1505, 1368, 1193, 1113, 1015, 904, 815. EI-MS m/z; 522 (m⁺), 480, 438, 396, 354. On the EI-MS of the acetylated compound, four acetyl groups were observed. The structure of compound I was thus identified as variegatic acid (3,3',4,4'-tetrahydroxy puluvinic acid) (Fig. 1).

Variegatic acid has been reported as the pigment responsible for the blueing of fresh sporophore caused by bruising from the fungus *Suillus variegatus (Swartz ex Fr.)* (Edwards and Elsworthy, 1967) and other *Boletus* species (Beaumont et al., 1968). It is a pulvinic acid derivative and Bresinsky and Rennschmid (1971) reported a chemotaxonomic relationship between leitpigment and *Suillus* section. But variegatic acid has not been reported as an antioxidant.

Identification of compound II

From the lyophilized powder of 23 kg fresh sporophore, 1200 mg of orange-red crystals were obtained from acetic acid. The melting point was 134~139°C (decomp.). From elemental analysis, the molecular formula of compound II was $C_{52}H_{70}O_8$ (Found; C, 75.6; H, 9.0; O, 15.4 Calculated; C, 75.9; H, 8.6; O, 15.6). The structure of compound II was suggested to be diboviquinone-4,4 (Fig. 2).

Beaumont and Edwards (1969) reported a benzoquinone, bovinone (2,5-dihydroxy-3-geranylgeranyl-1,4-benzoquinone) from *Suillus bovinus* and also the derivatives of bovinone, boviquinone-3 (2,5-dihydroxy-3-farnesyl-1,4-benzoquinone), dibovi-quinone-3,4-methylenedibovi-quinone-3,3 from *Gomplidius rutilus Fr.* and diboviquinone-4,4 from *Suillus bovinus* (Beaumont and Edwards, 1971). Boviquinone-3 has been reported from *Chroogomphus helveticus* and *Chroogomphus rutilus* by Steglich et al. (1971). Compound II was considered bovinone or diboviquinone-4,4 from spectroscopic measurements. Beaumont and Edwards (1969) reported that from fresh sporophores, they could obtain much bovinone but from the dried material, it was difficult to isolate pure bovinone. The dimerization of benzoquinone takes place in the presence of acid (Dean et al., 1955). Beaumont et al. (1971) treated boviquinone-4 with methanolic hydrogen chloride and reported it to be converted to a red polymeric oil and no diboviquinone was isolated. The absence of a quinone nuclear proton in the ^{1}H-NMR at δ5.98 and diboviquinone from *Suillus bovinus* consisted entirely of the symmetrical-4,4 compound (Beaumont et al., 1971) confirmed compound II to have the structure of diboviquinone-4,4. Spectroscopic data were as follows: IR ν_{max} (KBr disk) cm^{-1}; 3320, 2980, 2940, 2870, 1620, 1612, 1320, 1296, 1252, 1115, 782 ^{1}H-NMR δ(CDCl$_3$); 7.78 (4H, s), 5.15~5.07 (8H, m), 3.11 (4H, d),

A

B

Storage Days
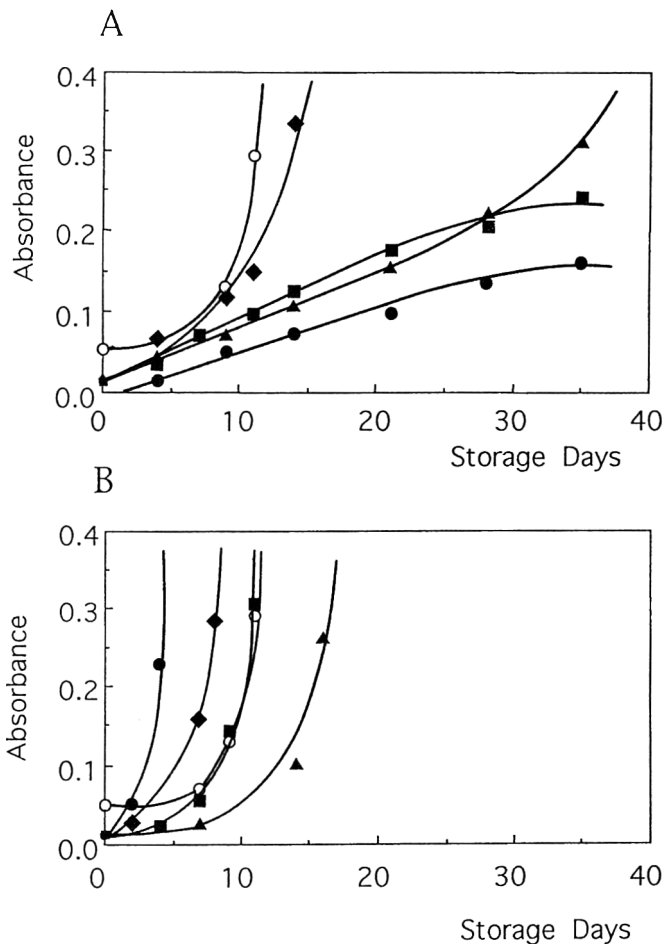
Fig. 3—Changes in antioxidative activity of isolated compounds in an emulsion system. ○ Control; ◆ Toc; ■ BHA; ● Compound I; ▲ Compound II. (A) Each compound was 5 mg/100 mL in the emulsion. (B) Each compound was 0.05 mg/100 mL in the emulsion. POV was measured by the thiocyanate method.

2.06~1.96 (24H, m), 1.72 (6H, s), 1.67 (6H, s), 1.59~1.55 (18H, m), ¹³C-NMR δ(CD₃ OD); 16.0 (C-26), 16.2 (C-25), 16.5 (C-21), 17.7 (C-16), 21.6 (C-7), 25.7 (C-11), 26.5, 26.6, 26.8 (C-12,17,22 or C-10,15,20), 39.7 (C-10,15,20 or C-12,17,22), 115.1 (C-24), 115.8 (C-9,14,19), 119.1 (C-23), 124.0, 124.2, 124.4 (C-8,13,18), 131.3 (C-3), 135.0 (C-6 or C-1,4 or C-2,5), 135.1 (C-1,4 or C-2,5), 137.5 (C-2,5 or C-1,4 or C-6), EI-MS m/z; 677, 468, 412 (M⁺ Bovinone), 343, 275, 233, 204, 193, 119.

The benzoquinone derivative was synthesized because of its scavenging activity of active oxygen species by Okamoto et al. (1982) and Okawa et al. (1991). We found diboviquinone to be an antioxidant.

Test for antioxidant activity

Antioxidant effectiveness of the isolated compounds and known standards was ascertained by three methods. Antioxidant activity in the oil phase for methyl linoleic acid was measured (Table 1). Both compounds I and II showed antioxidative activity, with compound I having the greater activity. The activity of compound I was greater than that of tocopherol and almost equal to BHA.

The time-course of autooxidation of linoleic acid in an emulsion was followed by the ferric thiocyanate method (Fig. 3). When concentrations of the compounds were 5 mg/100 mL in an emulsion, compound I showed the strongest antioxidative activity, followed by BHA and compound II. But at 0.05 mg/100 mL, only compound II was slightly stronger than the con-
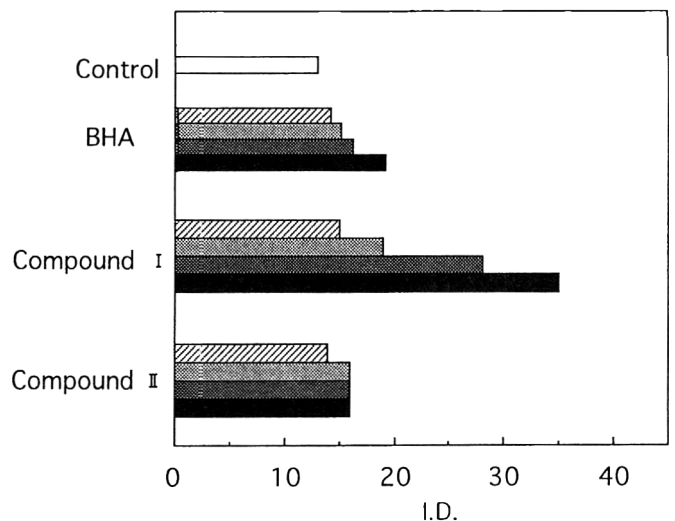
Fig. 4—Antioxidative activity of isolated compounds by weighing method. □ Control; ▨ 0.01; ▨ 0.02%; ■ 0.05%; ■ 0.1%. I.D. = Days required to reach 0.5% gain in weight by Weighing Method on soybean oil.

trol. Compound I and tocopherol had less antioxidative activity than the control and BHA the same. This indicated that compound I had strong antioxidative activity, but at the low concentration, it may act as a pro-oxidant.

Antioxidative activity was also shown (Fig. 4) by the induction period of a soybean oil to reach 5% weight gain. The induction period of compound I increased considerably with concentration. The activity of compound I was two times that of BHA at 0.05 and 0.1%. The induction period of compound II indicated antioxidant activity to be constant regardless of increased concentration.

It appears that compound I has strong antioxidative activity in an emulsion system and oil system, and compound II in an emulsion system. Thus, variegatic acid and diboviquinone make a relatively large contribution to the antioxidative activity of *Suillus bovinus*. There may be other compounds which could contribute to antioxidant activity. Further research is needed to isolate and identify compounds and estimate individual contributions to antioxidative activity. Such components may provide a basis for natural antioxidants in formulated foods.

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Decreasing Lipid Oxidation in Soybean Oil by a UV Absorber in the Packaging Material

M.A. PASCALL, B.R. HARTE, J.R. GIACIN and J.I. GRAY

——————————— ABSTRACT ———————————

The stability and effectiveness of Tinuvin 326™, a UV absorber, dispersed within the regrind layer of coextruded, multilayered, polypropylene-based containers to protect packaged, bleached soybean oil from photooxidation was investigated. The level of Tinuvin 326 in the containers was determined by using high pressure liquid chromatography (HPLC) and UV spectrophotometric methods. No loss of Tinuvin 326 from the containers was observed over a 42-day storage period at 21 and 35°C, respectively. The migration of Tinuvin 326 from the containers to the oil stored at 35°C was greater than at 21°C. Migration levels at both temperatures, however, were too low to notably reduce Tinuvin 326 levels in the containers. Bleached oil in containers with 0.3% Tinuvin 326 underwent less photooxidation than oil in containers with no Tinuvin 326 when exposed to fluorescent light at 21 and 35°C for 35 days.

Key Words: soybean oil, lipid oxidation, UV absorber, transparent packaging

INTRODUCTION

OXIDATIVE RANCIDITY OF OIL occurs when oxygen reacts with unsaturated fatty acids either spontaneously on exposure to air (autoxidation) or in the presence of light and a sensitizer (photosensitized oxidation) such as chlorophyll or myoglobin (Love, 1992). Frankel (1984) described this latter process as activation of oxygen to the singlet state by transfer of energy from the photosensitizer. The resulting singlet oxygen is extremely reactive. Such reactive oxygen can combine with carbons on either side of the double bond of a fatty acid molecule to yield an allylic hydroperoxide in the trans configuration. These hydroperoxides are relatively unstable and decompose to shorter chain carbon compounds such as fatty acids, aldehydes, and ketones which can cause food spoilage.

Fluorescent light can initiate photooxidation in foods (Sattar et al., 1976; Emmons et al., 1986; Luby et al., 1986). Therefore, much research has been directed toward development of barriers to light including metal, paperboard, colored glass and pigmented plastics (Sattar et al., 1976; deMan, 1978; Hoskin, 1988; Nkpa et al., 1990). In addition to light barriers, use of UV stabilizers for minimizing photooxidation has been reported. Such compounds perform this function by absorption of incident UV energy and concomitant quenching of photoexcited chromophores. Ultraviolet stabilizers are colorless or nearly colorless organic substances which can protect polymeric and other light-sensitive materials from degradation by sunlight and other sources of UV radiation (Hawkins, 1984). Several different classes of compounds have been used commercially to retard light-induced polymeric degradation, including UV absorbers, hindered amines, nickel chelates, hindered phenols and aryl esters (Dexter, 1984). Incorporated into transparent plastics, such compounds could be expected to impart increased stability to polymeric structures, and at the same time, provide them with the ability to block transmission of incident UV radiation. Crompton (1971) reported that the actinic effects of such un-

impeded radiation could cause discoloration of both the plastic material and the packaged foodstuff.

Tinuvin 326 2-(3'tert-butyl-2'-hydroxy'5'-methylphenyl)-5-chlorobenzo-triazole, is an ultraviolet absorber. Its incorporation into transparent polypropylene containers decreased vitamin A loss in milk exposed to incident UV radiation (Fanelli et al., 1985; Shipe et al., 1983). Therefore, its incorporation into containers used to package oils may result in increased product stability. Tinuvin 326 was approved by the FDA as a U.S. food packaging additive in April 1981 (Title 21 of the code of Federal Regulations).

Our specific objectives were: (1) to develop a methodology for quantitation of Tinuvin 326 in test containers; (2) to evaluate the stability of Tinuvin 326 in such materials in the absence of light, and at two storage temperatures; (3) to investigate the migration of Tinuvin 326 from the package material to the product (soybean oil); and (4) to determine any changes in product quality as related to Tinuvin 326 concentration (in the container wall), exposure to light, time, and storage temperature.

MATERIALS & METHODS

Test materials

The test packages were transparent, coextruded, multilayered polypropylene cups (Ball Plastics, Evansville, IN) with a volume of 130 mL and sidewalls of 0.35 mm thickness. The material profile of the containers from outside to inside was: polypropylene (95.3 μm)/regrind 80.0μm/adhesive 9.5μm/44 mol. % ethylene vinyl alcohol copolymer 15.2μm/adhesive 9.5μm/regrind 80.0μm/polypropylene 91.4μm. The control batch of containers had none, while the test batch had 0.3% Tinuvin 326 (Ciba Geigy, Hawthorne, NY) incorporated into the regrind layers. Alusuisse™ "Easy Peel" lid stock (Ball Plastics, Evansville, IN) was made of an aluminum foil/polypropylene laminate.

Sample preparation

Refined soybean salad oil, containing no added antioxidants (as indicated on the label), was obtained from the General Food Stores, Michigan State Univ. The oil was bleached by filtering (Vianni, 1980) through a chromatography column (4.5 cm internal diameter × 50 cm length). The column was packed from bottom to top with 2.0 cm glass wool, 2.0 cm MN-Kieselguhr G-HR (Macheney, Nagel, Germany), and a 100g mixture of 50% activated carbon Darco S-51 grade (American Norit, Jacksonville, FL), 35% MN-Kieselgel G-HR and 15% Florisil (Fluka Chemical, Ronkonkoma, NY). Finally, two successive layers of silicic acid (Fluka Chemical, Ronkonkoma, NY) and anhydrous sodium sulfate (Mallinckrodt, Paris, KY), each 1.0 cm, were added to complete the packing. All solvents used were analytical grade and were purchased from EM Science, Gibbstown, NJ. After an initial pass of 100 mL of hexane through the packed column, a mixture of 100g soybean oil and 150 mL hexane was eluted followed by 150 mL hexane. From the eluent the hexane was evaporated by using a Buchi rotary evaporator (Westbury, NY) under low vacuum at 30°C. The oil was transferred to an amber-colored glass bottle, flushed with nitrogen and stored in a freezer at −20°C. The columns were regenerated for reuse by eluting them with 150 mL of anhydrous ethyl ether, acetonitrile, methanol, acetonitrile, anhydrous ethyl ether and, finally, hexane in successive order. This was done after each batch of oil was bleached.

Tinuvin 326 extraction

The walls of sample containers were cut into pieces, ≈1 cm², and an average of 3.0g was used for each analysis. These were obtained after

removing the base and the upper heat sealable lip from each container. These showed differences between thicknesses and walls of each container. Intact containers had an average weight of 6.304g. Extraction of Tinuvin 326 was by Soxhlet with 110 mL HPLC-grade acetonitrile (Fisher, Pittsburgh, PA) for 18 hr. The extract was made up to 200 mL with acetonitrile in a volumetric flask. Each sample was extracted with 3 more aliquots of acetonitrile, which were also made up to 200 mL. The efficiency of extraction was investigated by obtaining UV absorbance spectra of sample container walls before and after Soxhlet extraction, using a Perkin-Elmer (Oak Brook, IL) Lambda 3B UV-visible spectrophotometer equipped with integrating sphere. Three samples ($\approx$3.0 cm $\times$ 1.5 cm) from each of the two types of containers (0.0 and 0.3% Tinuvin 326), and three from Soxhlet extractions, were mounted directly into the sample holder of the Integrating Sphere and their spectra determined.

Quantitation of Tinuvin 326 by HPLC

The Tinuvin 326 (acetonitrile extract) was quantitated by reverse phase HPLC. The HPLC system was equipped with a Perkin-Elmer (Oak Brook, IL) stainless steel column (0.24 cm i.d. $\times$ 25 cm length), with ODS-HC Sil-x-1 packing material. The mobile phase was acetonitrile: water (85:15, v/v) at a rate of 1 mL/min. The volume injected was 10 μL. The HPLC system consisted of a Perkin-Elmer Series 3B solvent delivery system and a LC-100 column oven, with a Perkin-Elmer LC-75 spectrophotometric detector set at 347.3 nm. The detector was interfaced to a Spectra Physics SP4200 Computing Integrator (Norwalk, CT) for quantification. Two replicates were taken at each observation, and each replicate was analyzed twice. Totals and averages for each replicate, provided the average peak areas for each sample which were analyzed. The concentration of Tinuvin 326 in the containers was determined from a standard curve developed by analyzing Tinuvin 326 solutions of known concentration in acetonitrile.

Quantitation of Tinuvin 326 by UV spectrophotometry

A Perkin-Elmer Lambda 4B Double Beam UV visible spectrophotometer (Norwalk, CT) was used to measure the Tinuvin 326 content of the acetonitrile extracts at wavelength 347.3 nm. Concentration of Tinuvin 326 in the containers was determined from standard curves developed by analyzing Tinuvin 326 solutions of known concentrations in acetonitrile.

Loss of Tinuvin 326 from containers

The rate loss of Tinuvin 326 from the test containers was determined using both HPLC and UV spectrophotometric methods, by monitoring

the change in Tinuvin 326 in the containers over a 42-day period, at ambient temperature (21 $\pm$ 1°C) and 35 $\pm$ 1°C. The containers were stored in the dark.

Migration of Tinuvin 326 into the oil

The migration of Tinuvin 326 from the containers was studied over a 42-day period at 21 $\pm$ 1°C and 37 $\pm$ 1°C. Samples (30) were filled with 120 mL of refined soybean oil, covered and heat sealed with the Alusuisse™ "Easy Peel" lid stock, by using a blister-pack sealer (Alloyd, Dekalb, IL), at 182.2°C and 275.8 KPa for 10 sec. Two oil-filled containers were removed weekly from storage, and the concentration of Tinuvin 326 in the oil was determined by HPLC, over the 42-day period. The container material was also analyzed for Tinuvin 326 using UV spectrophotometric and HPLC methods over the same 42 days.

The migration of Tinuvin 326 into the oil was evaluated by extracting 3 mL of oil with 15 mL acetonitrile in a 50 mL separatory funnel. After 30 sec of shaking, the upper acetonitrile layer was transferred to a sealed 100-mL glass centrifuge tube. The oil was extracted three more times, each time using a fresh aliquot of acetonitrile. The extracts were then combined and centrifuged (International Centrifuge, Model CM, Size 1, Boston, MA) at 2000 rpm for 15 min to effect further separation of any residual oil. The acetonitrile layer ($\approx$75 mL) was then concentrated to 5 mL by using a Buchi rota-evaporator (Flawil/Schweiz, Switzerland). The concentrate was then analyzed for Tinuvin 326 by HPLC.

The efficiency of extraction method was determined by adding Tinuvin 326 to soybean oil to produce a concentration of 40 mg/kg Tinuvin 326/oil. The Tinuvin 326 was extracted as described, and quantified by using the HPLC method. This was repeated three times. Quantities obtained were compared to initial amounts, and average differences served as the basis of recovery efficiency. The efficiency of the concentration step was similarly evaluated.

Light-inducted oxidation of soybean oil

Ninety 0% and ninety 0.3% Tinuvin 326 containers were heat sealed, as described after 15 mL of bleached soybean oil were added to each container. These were randomly divided into two groups, with each group having equal numbers of 0.0% and 0.3% Tinuvin 326 containers. One group was exposed to fluorescent light (Phillips, cool white, 120 volt $\times$ 34 watts and 120 cm in length) at 21°C, and the other at 35°C for a total of 35 days. The samples were illuminated in light boxes (91 cm $\times$ 61 cm $\times$ 53 cm) at an intensity of 538 Lux (General Electric, model J-55, light meter), and a distance of 64 cm from the light source. This light intensity was based on a survey of grocery aisles in several retail food stores in the East Lansing, MI area. At predetermined times (days 1, 2, 4, 7, 14, 21, 25, 28, 32, and 35), two samples from each
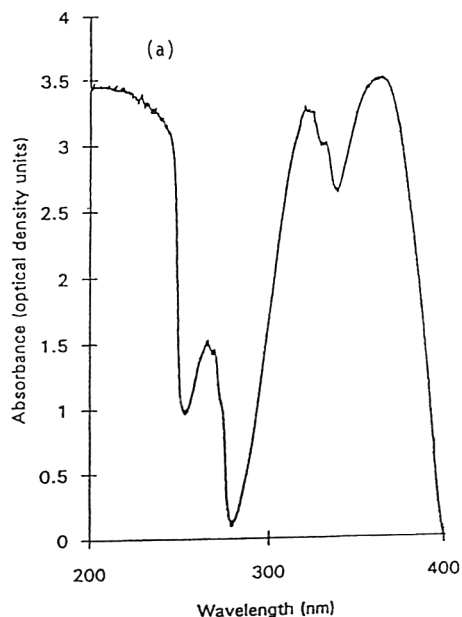
Fig. 1a—Absorbance spectrum of container wall before Soxhlet extraction to remove Tinuvin 326, using Acetonitrile as solvent.
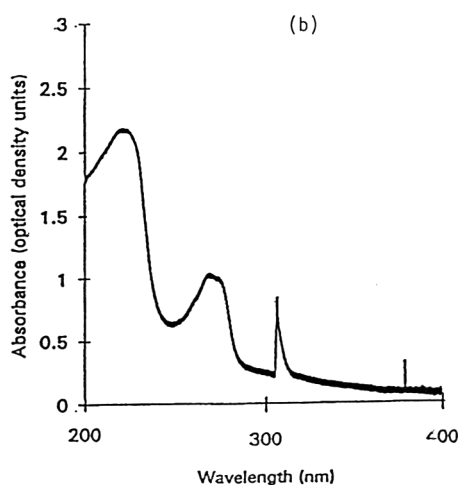
Fig. 1b—Absorbance spectrum of container wall after Soxhlet extraction to remove Tinuvin 326, using Acetonitrile as solvent.
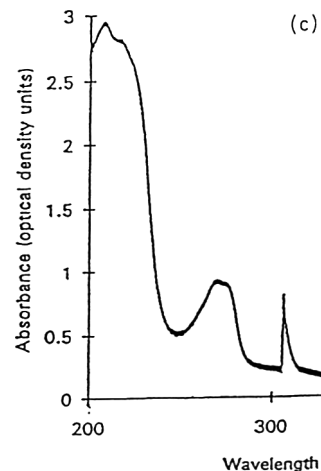
Fig. 1c—Absorbance spectrum of container wall with 0.0% Tinuvin.
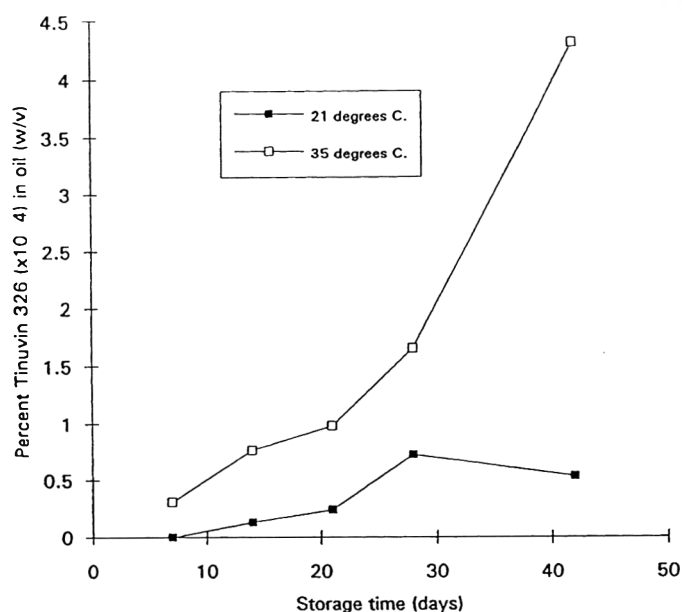
Fig. 2—Percent Tinuvin 326 migrating from containers to the packaged soybean oil using the HPLC method of analysis.

Fig. 3—Peroxide values of soybean oil stored at 21 and 35°C, in containers with 0.0% and 0.3% Tinuvin 326.

Table 1—Weight of Tinuvin 326 left in containers and weight migrated to stored packaged soybean oil (120 mL) using HPLC

Storage time (Days)	Wt g Tinuvin in oil ($\times 10^{-5}$)		Wt g Tinuvin in containers ($\times 10^{-3}$)	
	21°C	35°C	21°C	35°C
0	—	—	9.009	9.009
7	0.0	3.750	10.27	9.520
14	1.55	9.050	9.791	9.911
21	2.80	11.75	9.567	9.600
28	9.30	19.85	9.365	9.790
42	6.40	35.80	9.968	9.113

container type were removed, from each temperature, and the extent of lipid oxidation in the oil determined by the peroxide value method (AOAC, 1984). All measurements were duplicated and values reported as averages. The data were statistically analyzed by analysis of variance, using a complete randomized design, and computed using the MSTAT 4.0 statistical software package (Michigan State Univ., 1987).

RESULTS & DISCUSSION

Extraction of Tinuvin 326 from containers

The absorbance spectra of the walls of the two types of containers before and after extraction with acetonitrile were compared (Fig. 1a and 1b). Comparison of these spectra with that of the container wall (Fig. 1c) shows that the dispersed Tinuvin 326 in the container wall was quantitatively removed during Soxhlet extraction.

Similar spectra were obtained for solutions of acetonitrile alone and with 10 µg/ml Tinuvin 326. Dexter (1984) reported similar results for 2-(2'hydroxy-5-methylphenyl)benzotriazole in chloroform. This method was also used by Yshkevichyute and Shlyapnikov (1967) to quantify Tinuvin 326 in polyethylene material using heptane as solvent.

Loss of Tinuvin 326 from container to the atmosphere

The rate of loss of Tinuvin 326 from the test containers was determined as a function of storage time and temperature by using both HPLC and UV spectrophotometric methods. A concentration of 0.30 ± 0.006% Tinuvin 326 was obtained using the HPLC method at time zero. After 42 days storage, concentrations were 0.30 ± 0.003% at 21°C and 0.31 ± 0.001% at 35°C. Using the UV spectrophotometric method, an initial con-

centration of 0.31 ± 0.011% was obtained. After 42 days storage, concentrations were 0.31 ± 0.001% at 21°C and 0.32 ± 0.002% at 35°C. Thus, results showed little or no loss of Tinuvin 326 from the containers at either temperature during storage 42 days in absence of light. Yushkevichyute and Shlyapnikov (1967) determined levels of Tinuvin 326 (in heptane solutions) in 0.16 mm thick polyethylene material. Their findings showed an increased migration of Tinuvin 326 from the interior to the surface of the film at high temperatures, which stabilized after ≈400 hr, at 20, 30, and 40°C. The differences in those results and ours may be attributed to differences in thickness of the plastic, the nature of the laminate material and the manufacturing process. In our study, the Tinuvin 326 was dispersed into the regrind layers of the laminate and not directly exposed at the surface, as it was in the former study.
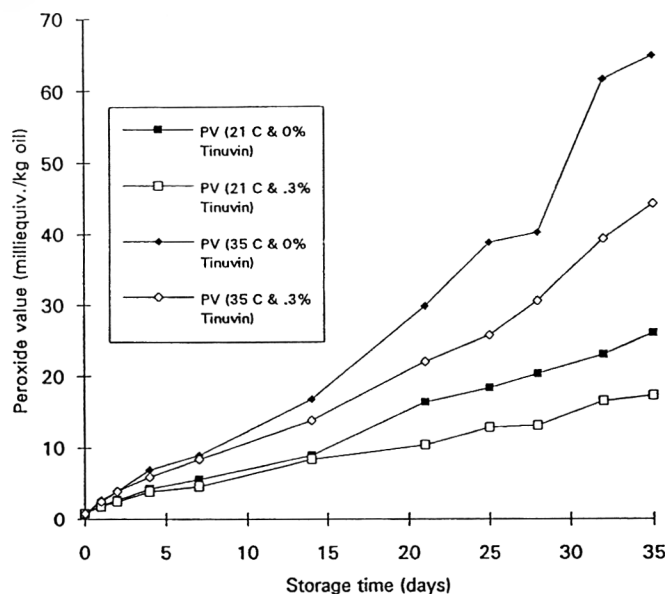
Soxhlet extraction efficiency was determined by both HPLC and UV spectrophotometric procedures. An average of 68.5% of the total level was extracted by the first Soxhlet extraction, 21.8% with the second extraction and 9.72% with the third extraction. A fourth Soxhlet extraction produced 0.00515% Tinuvin 326. Due to the low concentration of Tinuvin 326 in the fourth extract, three separate extractions were used to remove Tinuvin 326 from the material for subsequent quantifications.

Migration of Tinuvin 326 from container wall into oil

The amounts of Tinuvin 326 which migrated from the container wall into the oil at 21 and 35°C (HPLC method) were compared (Fig. 2). The weights (g.) of residual Tinuvin 326 in the containers and in the 120 mL soybean oil at the storage times and temperatures were also compared (Table 1). These results showed undetectable loss of Tinuvin 326 to the atmosphere and little migration of Tinuvin 326 from the containers to the storage oil. The percent recovery for extraction of Tinuvin 326 from the oil samples was 97.8%, whereas that for the rotaevaporation concentration averaged 81.0%. The level of migration of Tinuvin 326 from the container wall to the oil was higher at 35°C than at 21°C. At both temperatures however, the level of Tinuvin 326 in the containers remained relatively constant. Quantitatively, a low level of Tinuvin 326 migrated from the containers to the oil, especially at 21°C. This level was so low that during the first week of storage (21°C), Tinuvin 326 was not detected in the oil. The maximum quantity transferred to the oil was 4.8% of the initial level present in the containers after 42 days at 35°C.

Oxidation of soybean oil

Peroxide values for the oil in both sets of containers slowly increased during the first 14 days storage (Fig. 3). After that time, however, the oil in the containers with no Tinuvin 326 oxidized at a faster rate. Analysis of variance showed that there was a significant difference (P < 0.01) between peroxide values of oil stored in the containers with Tinuvin 326, and those for oil stored in containers without Tinuvin 326.

The trends obtained at 35°C were similar to those at 21°C. Shipe et al. (1983) and Fanelli et al. (1985) showed that when Tinuvin 326 was impregnated into plastic containers, a reduction in vitamin A (light sensitive) loss resulted. This protection could be attributed to the UV absorbent characteristic of Tinuvin 326. Guillet (1972) stated that UV absorbers could preferentially absorb light and harmlessly dissipate its energy. Tinuvin 326 absorbs light energy between 190–390 nm (ultra violet range) which may explain why it decreased photooxidation in oils and other light-sensitive foods.

CONCLUSIONS

NO DIFFERENCES were found in the levels of Tinuvin 326 in containers before and after storage in the dark at room (21°C) and elevated temperatures (35°C). A relatively low level of migration occurred of Tinuvin 326 into packaged soybean oil. Thus Tinuvin 326 is stable within the polymeric material for at 42 days or longer. The enhanced stability of soybean oil in containers with Tinuvin 326, indicated that such packaging systems could be used to reduce deterioration of vegetable oils during storage. Incorporation of Tinuvin 326 into the plastic did not noticeably change its transparency. A transparent polymeric material could thus be used to display a light sensitive product and at the same time protect it from incident ultra violet radiation.

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Heat Stability of Oil-in-Water Emulsions Containing Milk Proteins: Effect of Ionic Strength and pH

JOSEPHINE A. HUNT and DOUGLAS G. DALGLEISH

─────────── ABSTRACT ───────────

Emulsions (20 wt% soybean oil; 2 wt% protein) made with caseinate at pH 7 and with whey protein isolate (WPI) at pH 7 and 3 were stable to heating at 90 and 121°C. WPI emulsions destabilized at pH values between 3.5 and 4.0. In the presence of KCl (12.5—200 mM), large particles were formed in WPI emulsions at pH 3 and the emulsions were viscous. At pH 7, moderate concentrations of KCl decreased the heat stability and gels were formed. KCl had less effect on WPI emulsions made at pH 3. Combining the emulsions with caseinate allowed some control of the heat-induced gelation.

Key Words: emulsion, whey-protein isolate, heat stability, casein, oil-in-water

INTRODUCTION

MANY FOOD PRODUCTS based on emulsions are subjected to heat treatment prior to distribution. The heat treatment is applied generally to extend the microbiological shelf-life of the product, but may be moderated if the heat treatment destabilizes the emulsion. The effect of heating at 90°C on emulsions made with WPI has been reported (Jost et al., 1986; Masson and Jost, 1989; Yost and Kinsella, 1992). It was demonstrated that gels may be formed under those conditions. Such gels were produced at much lower concentrations than would be required if the protein was present alone in solution (Matsudomi et al., 1992). It was suggested, therefore, that the emulsified droplets became incorporated into the gel matrix. The formation of gels depends greatly on variables such as ionic strength and pH (Mulvihill and Kinsella, 1987). Therefore, to investigate further the formation of such gels during heating, we introduced both pH and KCl concentration as variables. KCl was used in preference to NaCl since it is a more acceptable food additive. Another variable in emulsions made with proteins is the distribution of the protein. It may either be adsorbed at the surface of the emulsion droplet or it may be free in solution. In addition, it is possible to alter the composition of both adsorbed and free protein by adding a second protein after homogenization (Hunt and Dalgleish, 1995). Our objective was to study the effect of such exchange experiments on heat stability of emulsions. In addition to a heat treatment of 90°C for 30 min, which has been reported (Jost et al., 1986; Masson and Jost, 1989), we also introduced a second heat treatment comparable to conditions found during retort treatments of canned products.

MATERIALS & METHODS

Materials

Soybean oil, KCl and buffer salts (KH_2PO_4, K_2HPO_4, trisodium citrate) were purchased from Sigma Chemical Company, St. Louis, MO. Caseinate was prepared in the laboratory from skim milk by acid precipitation, washing and dissolving the precipitate with NaOH to pH 7.0, followed by freeze-drying (Fang and Dalgleish, 1993). WPI, prepared by ion-exchange from sweet whey, was provided by Protose Separations Inc., Teeswater, Ontario.

Emulsion preparation and determination of particle size

Oil-in-water emulsions (20 wt% soybean oil; 2.0 wt% protein) were prepared with caseinate or WPI at pH 7 (phosphate buffer, 20 mM,

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prepared by mixing solutions of 20 mM KH_2PO_4 and K_2HPO_4) and pH 3 (citrate buffer, 20 mM, prepared by titrating trisodium citrate with HCl) and various concentrations of KCl (0 mM to 200 mM), using a Microfluidizer M110S (Microfluidics Corp., Newton, MA) at an input pressure of 0.3 MPa (corresponding to a homogenizer pressure of 42 MPa). Protein solutions were filtered through 0.22 μm cellulose nitrate syringe filters (Millipore Ltd, Mississauga, Ontario, Canada) prior to homogenization. Oil (4g) was drawn into the Microfluidizer barrel and 16g of buffer solution containing appropriate amounts of protein was added to the reservoir. The pump was operated for 10 strokes with the sample recirculating each time through the reservoir, after which the contents of the reservoir were removed, stirred, replaced, and cycled for 10 more strokes of the pump to make the final emulsion.

The weight-average diameters ($d_{4,3}$) of the emulsion droplets and their size distribution (volume fraction as a function of particle size) were determined using a Mastersizer X (Malvern Instruments Ltd., Malvern, U.K.), with optical parameters (refractive index of the particles 1.43) defined by the manufacturer's presentation code 0303, and using the software supplied with the instrument. Water was used to disperse the emulsion, with a dilution factor of ≈ 1/1000. The water was deionized using a Milli-Q system (Millipore Ltd, Mississauga, Ontario, Canada).

Heat treatments

Emulsions were subjected to two different heat treatments: (a) 5 mL of emulsion was pipetted into a test-tube and heated in a water bath at 90°C for 30 min. Samples were left to cool at room temperature (≈23°C). This treatment was sufficient to completely denature the whey proteins; (b) 20 mL of emulsion was pipetted into a 50 mL Pyrex beaker and heated at 121°C for 10 min in an autoclave. After the exhaust cycle, samples were removed and left to cool at room temperature. This heat treatment was similar to many retort treatments in the food industry. Because the heating was by steam, the open beakers did not notably lose water (less than 5%) during the heating process. This was checked by weighing before and after heating.

Interchange experiments

In some experiments the protein composition of the serum phase of emulsions was changed to determine how this influenced emulsion stability and heat stability. The emulsion was first centrifuged at 15 000 × g for 1 hr at 25°C. Most droplets rose to form a layer at the top of the tube and the underlying serum phase was removed with a syringe. The upper layer of droplets was resuspended in a serum made up of either the original emulsion buffer, or a buffered solution containing additional protein, making sure that the volume fraction of the original emulsion was maintained (Dickinson et al., 1989). This mixed emulsion was left stirring for 1.5 hr, after which the average diameter and size distribution of the emulsion droplets, and the stability of the mixed emulsion to the 2 heat treatments, were determined. From earlier studies (Hunt and Dalgleish, 1994a,b) we knew the amount of protein which was in the serum of the original emulsion. The concentration of protein in the replacement serum was designed to equal that amount.

Replication of experiments

All experiments were repeated at least once, in some cases twice. In general replicates agreed very closely with one another in overall behavior and in size distributions of the particles. Average particle sizes differed by no more than 0.04 μm between replicates.

RESULTS & DISCUSSION

Effect of pH on heat stability

When emulsions (20 wt% soybean oil; 20 mM buffer salts) made with 2 wt% WPI at pH 7 and pH 3 were heated at 90°C for 30 min and 121°C for 10 min, no change in size distribution of emulsion droplets was observed. The emulsions were there-

fore considered stable. Caseinate emulsions (2 wt% protein, 20 mM buffer salts) made at pH 7 were also stable to both heating conditions.

It was not possible to make emulsions at pH 4 using WPI because of the very low solubility at that pH. Therefore, to determine the pH limit for emulsion stability, aliquots of an emulsion made at pH 3 with 2 wt% WPI were adjusted to different pH values between 3 and 4 by titration with small volumes of 1M NaOH (the volume required caused only insignificant dilution of emulsion). The effect of pH adjustment and heat treatment on size distribution of the emulsion droplets was compared (Fig. 1). As the pH of the emulsion approached 4, the emulsion became less stable, as evidenced by changes in size distribution. At pH < 3.5, there was no change in size distribution either before or after heating. However, at pH 3.6, although there was little change in size distribution of the unheated emulsion droplets, heating at 90°C for 30 min broadened the size distribution, and heating at 121°C produced a smooth spreadable paste which showed no signs of syneresis. At pH 3.8 and 4.0 the emulsions showed a broadening of the particle size distribution even before heating. Both heat treatments produced coarse self-supporting gels. In none of the emulsions did we find evidence for time dependent changes. Control (i.e., unheated) emulsions showed no changes in particle size during storage throughout 2 days. Likewise, although changes could be induced by heating, emulsions after heating showed no time-dependent changes.

Solutions of β-lactoglobulin form weak opaque gels in the region of the isoelectric point (pI), because protein-protein interactions are favored and protein aggregation is therefore very rapid. However, as the pH changes away from the pI (for α-lactalbumin, between pH 4.2 and 4.8, and for β-lactoglobulin about 5.2), charges on the protein molecules are increased. This slows the rate of aggregation since electrostatic repulsive forces must be overcome, and more structurally ordered, translucent gels are formed (Egelandsdal, 1980; Mulvihill and Kinsella, 1987; Stading and Hermansson, 1991). However, for emulsions made with whey proteins, the size of the protein-coated emulsion droplets is an important factor in determining whether gelation occurs during heating at 90°C for 30 min. Large droplets (>1000 nm) did not form gels, but smaller droplets (around 450 nm) produced gels. If the droplets were small enough, they could become incorporated into the gel matrix and facilitate gelation (Jost et al., 1986; Masson and Jost, 1989). Indeed, scanning and transmission micrographs of WPI gels containing emulsified butterfat droplets have shown that the droplets were intimately associated with the gelled protein matrix (Yost and Kinsella, 1992). Probably, at pH values close to the pI, two competing effects occur, namely (1) flocculation of emulsion droplets and (2) their incorporation into a gel formed with the whey protein in solution. The flocculation will increase the effective size of the dispersed phase and alter its incorporation into the gel matrix and, therefore, contribute to the changing appearance of the gels at pH values between 3.5 and 4.

It was not possible to solubilize caseinate at pH 3, but in an attempt to produce an emulsion at pH 3 one was first made at pH 7 and then titrated to pH 3. However, the emulsion did not stabilize after passing through the pI and remained in a curdled/aggregated state. In contrast, it was possible to titrate emulsions made with WPI through the isoelectric point from pH 3 to pH 7 and back without destabilizing the emulsion (Hunt and Dalgleish, 1994b), and the heat stability of such titrated emulsions was also retained.

Effect of KCl on heat stability

The presence of up to 200 mM KCl during the homogenization of emulsions made with caseinate had no effect on size distribution of emulsion droplets. Furthermore, heat stability of emulsions was not affected, i.e., no changes in the size distribution of the droplets were observed after the emulsion had been subjected to both heat treatments.
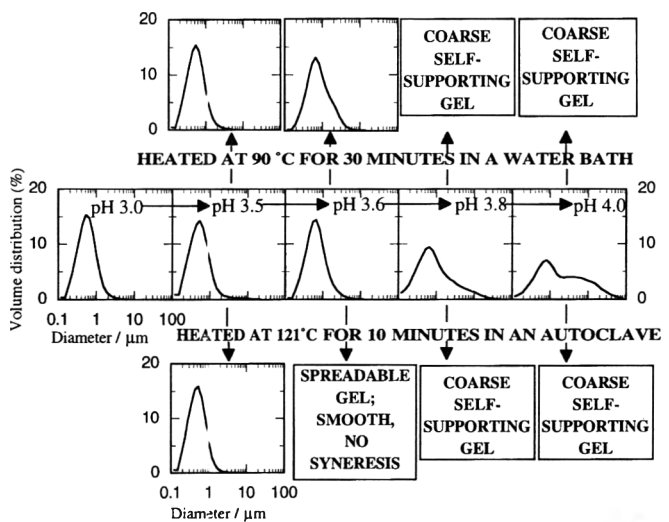
Fig. 1—Effect of pH adjustment and different heat treatments on the droplet size distribution of an emulsion (20 wt% soya oil, 2.0 wt% WPI) originally prepared at pH 3 in citrate buffer, and adjusted to higher pH values by the addition of NaOH. The small diagrams show the size distributions measured using the Mastersizer after each treatment.

Incorporation of KCl up to 25 mM during formation of emulsions containing WPI had little effect on particle size. However, at concentrations above that (up to 200 mM) the particle sizes in the emulsions increased. This effect was especially evident in emulsions prepared at pH 3 (Fig. 2). The size distribution in these latter cases was broadened considerably relative to the emulsions prepared using low concentrations of KCl (Fig. 3).

The addition of KCl, up to 50 mM, did not affect the heat stability of the WPI emulsions at 90°C and 121°C, at either pH 3 or pH 7 (Fig. 3 and 4, respectively). At pH 7 and a KCl concentration of 100 mM, the emulsion was stable to heating at 90°C, but it formed a weak gel when heated at 121°C. Increasing the KCl concentration to 200 mM produced a smooth, self-supporting gel when the emulsion at pH 7 was heated either at 90 or 121°C. Much larger values of the weight-average particle diameter ($d_{4,3}$) were observed for emulsion droplets made with high concentrations of KCl at pH 3, compared to pH 7 (Fig. 2), but the effect of KCl on heat stability of the emulsions at pH 3 was less than at pH 7. At pH 3, a gel was formed only during heating at 121°C in the presence of 200 mM KCl. This gel resembled cottage cheese and exhibited signs of syneresis. Heating at 90°C produced a very viscous, paste-like liquid in the emulsion at pH 3 containing 200 mM KCl. In the presence of 100 mM KCl, neither of the heat treatments had a marked effect on either the droplet size distribution or the macroscopic appearance of the emulsion at pH 3, except perhaps for a slight increase in viscosity (not quantified).

The emulsions which were prepared at pH 3 with added KCl were noticeably more viscous than those made at pH 7 at the same ionic strength. The high viscosity and broad distribution of particle sizes indicate that, even before heating, there was some interaction between emulsion droplets and/or unadsorbed protein. We assumed this to be a result of the reduction of charge repulsions by the higher ionic strength. However, the aggregation measured during formation of the emulsion at pH 3 in the presence of 100 or 200 mM KCl did not cause the emulsion to become unstable, since the size distribution of emulsion droplets did not change as a function of time.

In its simplest form, gelation is a two-stage process (Ferry, 1948). An initial denaturation and/or conformational change of native protein, induced by heat, produces protein molecules in various states of denaturation and which have, therefore, different functional properties. These may then associate through 'active sites' to form the gel matrix, providing that the balance between attractive and repulsive forces is favorable (Kinsella,
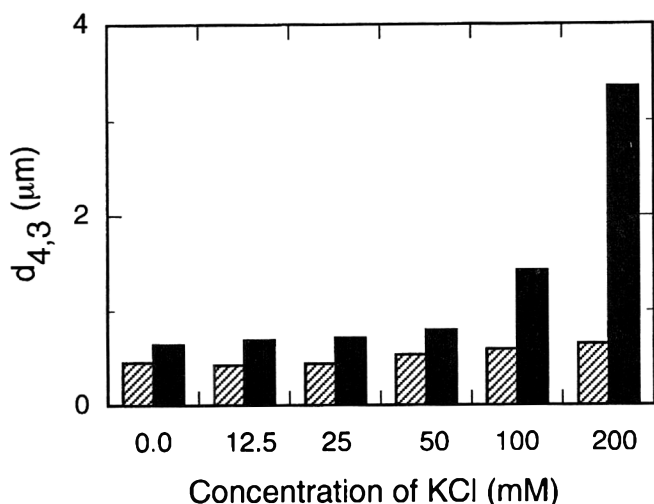
Fig. 2—Effect of the concentration of KCl on the average diameter ($d_{4,3}$) of droplets in emulsions (20 wt% soybean oil, 2.0 wt% WPI) prepared at pH 7 (hatched bars) or pH 3 (solid bars).
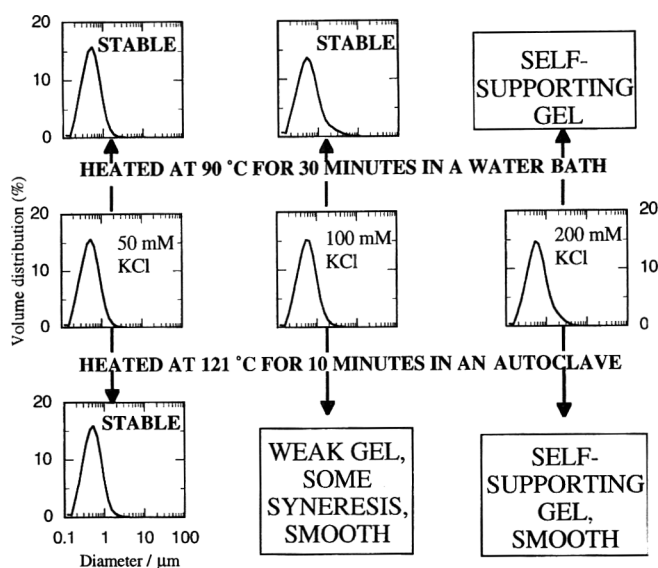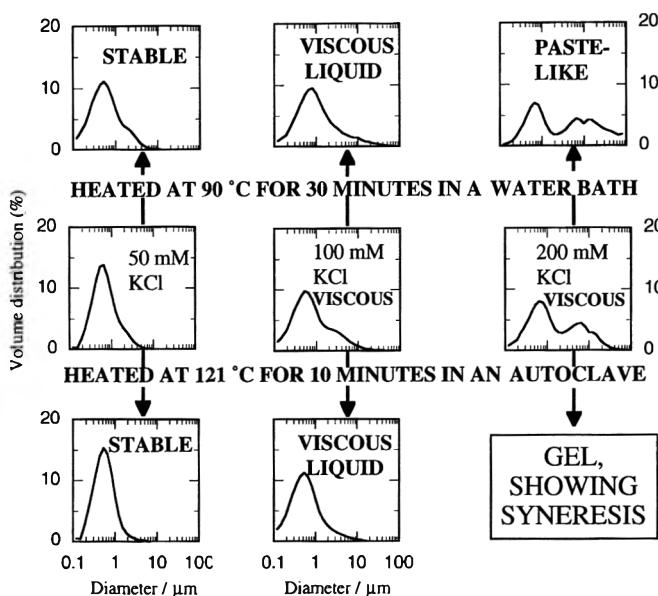

Fig. 3—Effect of KCl concentration and heat on the droplet size distribution of emulsions (20 wt% soybean oil, 2.0 wt% WPI), prepared at pH 3 in citrate buffer. Size distributions shown were measured using the Mastersizer.

Fig. 4—Effect of KCl concentration and heat on the droplet size distribution of emulsions (20 wt% soybean oil, 2.0 wt% WPI) at pH 7 in phosphate buffer. Size distributions shown were measured using the Mastersizer.

KCl. Therefore, despite the fact that KCl tended to increase the amount of adsorbed α-lactalbumin, there was always more unadsorbed α-lactalbumin in emulsions made at pH 7 than in those emulsions made at pH 3. This compositional difference may be important since the proportion of β-lactoglobulin to α-lactalbumin is known to affect gelation and gel strength (Matsudomi et al., 1992). Moreover, at pH 3 the α-lactalbumin would lose its central ion of calcium, and its conformation would be in the molten globule state, which may be more reactive than the native state (Dickinson and Matsumura, 1994).

Effect of changing protein composition of the serum on emulsion stability

It was suggested in the previous section that the composition of the unadsorbed protein in the serum phase may help to determine whether or not gelation occurs. To investigate this further, an emulsion containing 2 wt% WPI and 200 mM KCl was prepared and then the emulsion droplets were collected by centrifugation, the original serum phase was discarded, and the centrifuged emulsion droplets were resuspended either in buffer (phosphate at pH 7, 200 mM KCl) or a 1 wt% caseinate solution (phosphate at pH 7, 200 mM KCl). The original emulsion was chosen because it was known to form a gel when heated at both 90°C for 30 min and 121°C for 10 min. Also, in this emulsion we knew that the protein was divided about equally between adsorbed and unadsorbed states (Hunt and Dalgleish, 1994a). The effect of changing the serum composition on the stability of the emulsion was followed (Fig. 5). The original emulsion had a broad size distribution with a $d_{4,3}$ = 0.70 μm, and when the emulsion droplets were resuspended in buffer the size distribution remained broad and there was very little change in the $d_{4,3}$. However, when the cream was resuspended in caseinate solution, the size distribution became narrower and the $d_{4,3}$ was reduced to 0.54 μm. This was a result of caseinate adsorption at the surface of the emulsion droplet (Hunt and Dalgleish, 1995) which had the effect of breaking up flocs which had been present in the original WPI emulsion. Simply mixing the emulsion cream with caseinate (not re-homogenizing) could not have any effect in reducing sizes of the emulsion droplets. Thus, it is evident that the broad size distribution in the presence of KCl resulted from flocculation and not coalescence, which would not be reversed without re-homogenizing the sample.

Changing the serum composition altered the behavior of the emulsion. When the cream was resuspended in buffer, both heat

1984; Mulvihill and Kinsella, 1987). It is qualitatively apparent that the emulsions made at different pH values must contain different molecular species and different particles. Our results suggest that the "pre-aggregation" found during formation of the emulsion at pH 3 prevented further denaturation and aggregation by fixing the protein molecules in a stable unreactive conformation. The active sites which are necessary for gel formation may be either used up in forming the pre-aggregated structures, or are present in a different conformation which makes them unavailable to form junction points.

The composition of the unadsorbed protein may also be important. We have shown recently (Hunt and Dalgleish, 1995) that, in emulsions containing 2% WPI prepared at pH 7 in the absence of KCl, β-lactoglobulin and α-lactalbumin adsorbed in proportion to their concentrations in the WPI. The presence of KCl at concentrations >50 mM during homogenization somewhat decreased the proportion of adsorbed β-lactoglobulin and increased the proportion of adsorbed α-lactalbumin. In contrast, the surface composition of emulsion droplets stabilized by WPI at pH 3 was around 50% α-lactalbumin, at all concentrations of
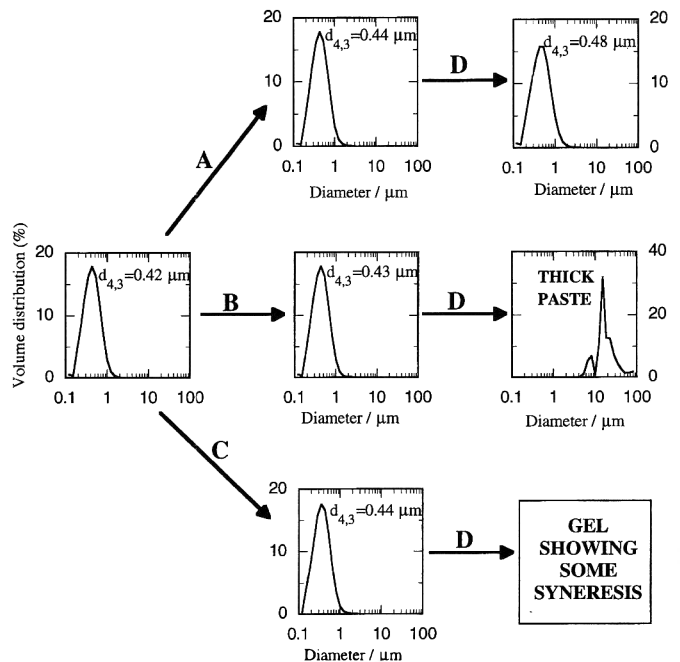
Fig. 5—Effect on the size distribution of particles in an emulsion (20 wt% soybean oil, 2.0 wt% WPI), prepared at pH 7 in phosphate buffer containing 200 mM KCl. The treatments were: (A) the cream was redispersed in buffer at pH 7 containing 200 mM KCl; (B) the cream was redispersed in the same buffer containing 1.0 wt% caseinate; (C) a heat treatment of 90°C for 30 min was given or (D) or a heat treatment of 121°C for 10 min was given.

Fig. 6—Effect on the size distribution of particles in an emulsion (20 wt% soybean oil, 2.0 wt% caseinate), prepared at pH 7 in phosphate buffer containing 200 mM KCl. The cream was redispersed in a solution at pH 7 containing 200 mM KCl and (A), 1.0 wt%; (B), 1.5 wt% and (C), 2.0 wt% WPI, before heating at 121°C for 10 min (Treatment D).

treatments resulted in broader size distributions and larger $d_{4,3}$ values, and, in the case of heating at 121°C for 10 min, a bimodal size distribution was evident. However, no gel was formed showing that unadsorbed WPI was essential to form the gel which was observed when the control emulsion was heated. This was in agreement with previous observations (Jost et al., 1986). When the WPI cream was resuspended in caseinate solution and then heated either to 90°C or 121°C, the size distribution of emulsion droplets did not change and the emulsion did not gel. In this particular emulsion, the composition of the adsorbed and unadsorbed protein had been changed. First, all of the unadsorbed whey protein was removed by re-dispersing the cream in caseinate solution. Some of the caseinate then became adsorbed (notably the α_{s1}-, α_{s2}- and β-caseins) and all of the adsorbed α-lactalbumin was displaced along with a portion of the adsorbed β-lactoglobulin (Hunt and Dalgleish, 1995). Therefore, by changing the distribution and composition of the protein we altered the behavior of the emulsion during heat treatment.

In a similar experiment, the cream of an emulsion made with 2 wt% caseinate at high ionic strength (200 mM KCl) was dispersed in buffered solutions of WPI of varying concentrations and a KCl concentration of 200 mM. Redispersing the caseinate cream in solutions of WPI up to 2.0 wt% had no effect (Fig. 6) on the size distribution of the emulsion droplets. This was expected, since we know (Hunt and Dalgleish, 1995) that no whey protein becomes adsorbed when the cream and protein solution are mixed. When these mixed emulsions were heated at 90°C for 30 min, no change in the size distribution was observed except for the case when the caseinate cream had been dispersed in 2.0 wt% WPI. Here the $d_{4,3}$ increased slightly from 0.44 to 0.48 μm. When the emulsions were heated at 121°C for 10 min, only the caseinate cream which had been redispersed in 1.0 wt% WPI remained stable, even though the $d_{4,3}$ increased slightly from 0.44 μm to 0.48 μm. The emulsion comprising caseinate cream + 1.5 wt% WPI resembled a thick paste after heat treatment, and when the cream was dispersed in 2.0 wt% WPI a gel was formed (Fig. 6). This gel exhibited signs of syneresis and was not as strong as the gel formed from a WPI emulsion under the same conditions of heating and ionic strength.

This experiment demonstrated clearly the importance of unadsorbed proteins in determining the heat stability of emulsions. Comparing the results of the caseinate and WPI emulsions, it is apparent that gelation is more likely when whey proteins constitute both the adsorbed and free protein. This suggests that, in the WPI emulsions, there was interaction between the adsorbed and unadsorbed whey proteins to facilitate gelation. It is also possible for caseins and whey proteins to interact through disulfide bridges, e g., κ-casein and β-lactoglobulin, during the heat treatment of homogenized milk (Sharma and Dalgleish 1993). However, the complexes between caseins and whey proteins are less active than complexes between whey proteins in forming gels. When the adsorbed protein is caseinate and the free protein is WPI, the concentration of unadsorbed whey protein has to be in excess of 1.5 wt% and the sample must be heated at 121°C for a gel to form at high ionic strength. However, a WPI emulsion (200 mM KCl) gels when the concentration of unadsorbed protein is only 1 wt%, even when heated at 90°C. Note that a 2 wt% solution of WPI does not gel under these same conditions of heat, pH and ionic strength. This shows that the emulsion droplets allow gels to be formed at much lower concentrations of WPI regardless of the protein which was originally used to make the emulsion droplets.

CONCLUSIONS

IN SOLUTION, whey protein concentrations of 8 % have been used to produce self-supporting gels. The limiting concentration of whey protein to produce self-supporting gels is known to be reduced to about 4—5% (with 20% oil) if an emulsion was formed first before the protein was heated to 90°C for 30 min. It is possible to produce gels at whey protein concentrations as low as 2 wt% using heat treatments at 90°C or 121°C and ionic strengths in excess of 50 mM. Emulsions made using caseinate did not form gels when heated, even at high ionic strengths. Furthermore, when the serum of WPI emulsions was replaced with a solution of caseinate at high ionic strength, gelation during heat treatment was prevented. Conversely, WPI addition to the cream of emulsions made with caseinate facilitated gel for-

Competitive Adsorption Between Sodium Caseinate and Oil-Soluble and Water-Soluble Surfactants in Oil-in-Water Emulsions

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——————— ABSTRACT ———————

Competitive adsorption between sodium caseinate and either a water-soluble surfactant, Tween 60 (polyoxyethylene sorbitan monostearate—PSM) or an oil-soluble surfactant, Span 60 (sorbitan monostearate—SM) was studied in oil-in-water emulsions. Surfactants were present during homogenization. Surface concentration of protein in freshly prepared emulsions decreased as concentration of PSM or SM increased. However, only partial displacement of protein was observed with either surfactant. The reduction in protein surface concentration was greater in the presence of PSM. Interfacial protein composition was independent of surfactant type. In the absence of surfactant, preferential adsorption of β-casein occured in emulsions containing ≤ 1.0 wt % protein. On addition of surfactant preference for β-casein at the interface was reduced.

Key Words: sodium caseinate, water-soluble surfactant, oil-soluble surfactant, monostearate, protein concentration

INTRODUCTION

BECAUSE OF ITS EXCELLENT EMULSIFYING PROPERTIES, sodium caseinate is commonly used in a wide range of emulsions in the food industry. These include coffee whiteners, cream liqueurs and whipped toppings. Sodium caseinate is composed of a mixture of casein molecules α_{s1}-, α_{s2}-, β- and κ- caseins (Swaisgood, 1992). Owing to the high surface activity of its constituent proteins, especially α_{s1}- and β-caseins, sodium caseinate is rapidly adsorbed at the oil-water interface during emulsification. This confers stability to the resultant emulsion from coalescence and/or flocculation (Dickinson, 1994).

Most formulated food emulsions contain not only milk protein but also small-molecule surfactants (emulsifiers) which compete for the emulsion interface during and after formation of the emulsion. The distribution of proteins and surfactants between the droplet surface and the bulk phase is an important factor affecting the stability, texture and ease of formation of an emulsion (Dickinson and Stainsby, 1982; Darling and Birkett, 1987; Goff and Jordan, 1987; Bergenstahl and Claesson, 1990; Barford et al., 1991; Krog, 1991).

Studies of competitive adsorption between milk proteins and surfactants in model emulsions have tended to focus on systems containing binary or ternary mixtures of individual milk proteins and surfactants with either hydrocarbon or purified triglyceride as the oil phase (de Feijter et al., 1987; Dickinson et al., 1989a, 1990, 1993; Courthaudon et al., 1991a,b,c,d; Dickinson and Tanai, 1992; Chen and Dickinson, 1993; Dickinson and Iveson, 1993). Such work has provided valuable information on the composition of the stabilizing layer around oil droplets in oil-in-water emulsions. However, it is much more relevant to the food industry to study systems containing more complex protein emulsifiers such as sodium caseinate.

Our objective was to investigate the competitive adsorption between commercial sodium caseinate and surfactants in a model oil-in-water emulsion. The surfactants studied were

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Tween 60 (polyoxyethylene sorbitan monostearate—PSM) and Span 60 (sorbitan monostearate—SM), which are water-soluble and oil-soluble, respectively. Both surfactants are commonly found in a wide range of food emulsions.

MATERIALS & METHODS

Materials

Spray-dried sodium caseinate (Alanate 180) was supplied by the New Zealand Dairy Board, Wellington, New Zealand. Polyoxyethylene sorbitan monostearate (PSM) and sorbitan monostearate (SM) were obtained from Sigma Chemicals, St. Louis, Mo, U.S.A. PSM has an estimated hydrophile-lipophile balance (HLB) of 14.4, and the estimated HLB of SM is 4.7 (Charalambous and Doxastakis, 1989). Soya oil was purchased from Davis Trading Co., Palmerston North, New Zealand.

Preparation of emulsions

Oil-in-water emulsions (20 wt% soya oil, deionized water, pH 7) were prepared at 50°C, using a two-stage Rannie homogenizer (Rohosmvej 8 DK-2620, Albertslund, Denmark). Operating pressures were 23 MPa in the first and 3 MPa in the second stage. Sodium caseinate in the concentration range 0.25–2.0 wt% of the final emulsion and PSM in the concentration range 0–1.0 wt% of the final emulsion were dissolved separately in deionized water at 50°C. Soya oil (at 50°C) and the solutions containing sodium caseinate and PSM were combined and mixed by a single pass through the homogenizer at atmospheric pressure prior to homogenization. Oil-in-water emulsions containing sodium caseinate and SM were prepared in a similar way, except SM (concentration range 0–1.0 wt% of the final emulsion) was dissolved in the oil at 50°C and combined with the sodium caseinate solution prior to homogenization. Levels of protein and surfactant were typical of those used in common food emulsions.

A Malvern Mastersizer MSE (Malvern Instruments Ltd, Worcs, U.K.) was used to determine the volume-surface average diameter ($d_{3,2}$) and the specific surface area (area/unit mass) for each emulsion. Analysis was carried out within 1 hr of initial preparation.

Surface protein concentration

Each emulsion was centrifuged at 45,000×g for 60 min at 20°C to separate the oil droplets from the aqueous phase. The aqueous phase was removed with a syringe, filtered through a 0.22 μm Millipore membrane filter, and the protein content determined by the Kjeldahl method with a Tecator Kjeltec system (Tecator AB, Hoganas, Sweden). A factor of 6.38 was used to convert nitrogen to protein content. The oil phase was kept for subsequent compositional analysis using sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE).

The surface concentration. Γ (mg protein/m²), of sodium caseinate was calculated from the surface area of the oil droplets determined by the Malvern Mastersizer and the difference in amount of protein used to prepare the emulsion and that measured in the subnatant after centrifugation.

Composition of interfacial protein

The interfacial composition of caseins was determined by a method similar to that described by Hunt and Dalgleish (1994). The cream separated from emulsions, following the centrifugation procedure. was washed with deionized water and recentrifuged at 45,000×g for 60 min at 20°C to remove unadsorbed protein. The separated cream from the second centrifugation was then spread onto filter papers (Whatman 50)
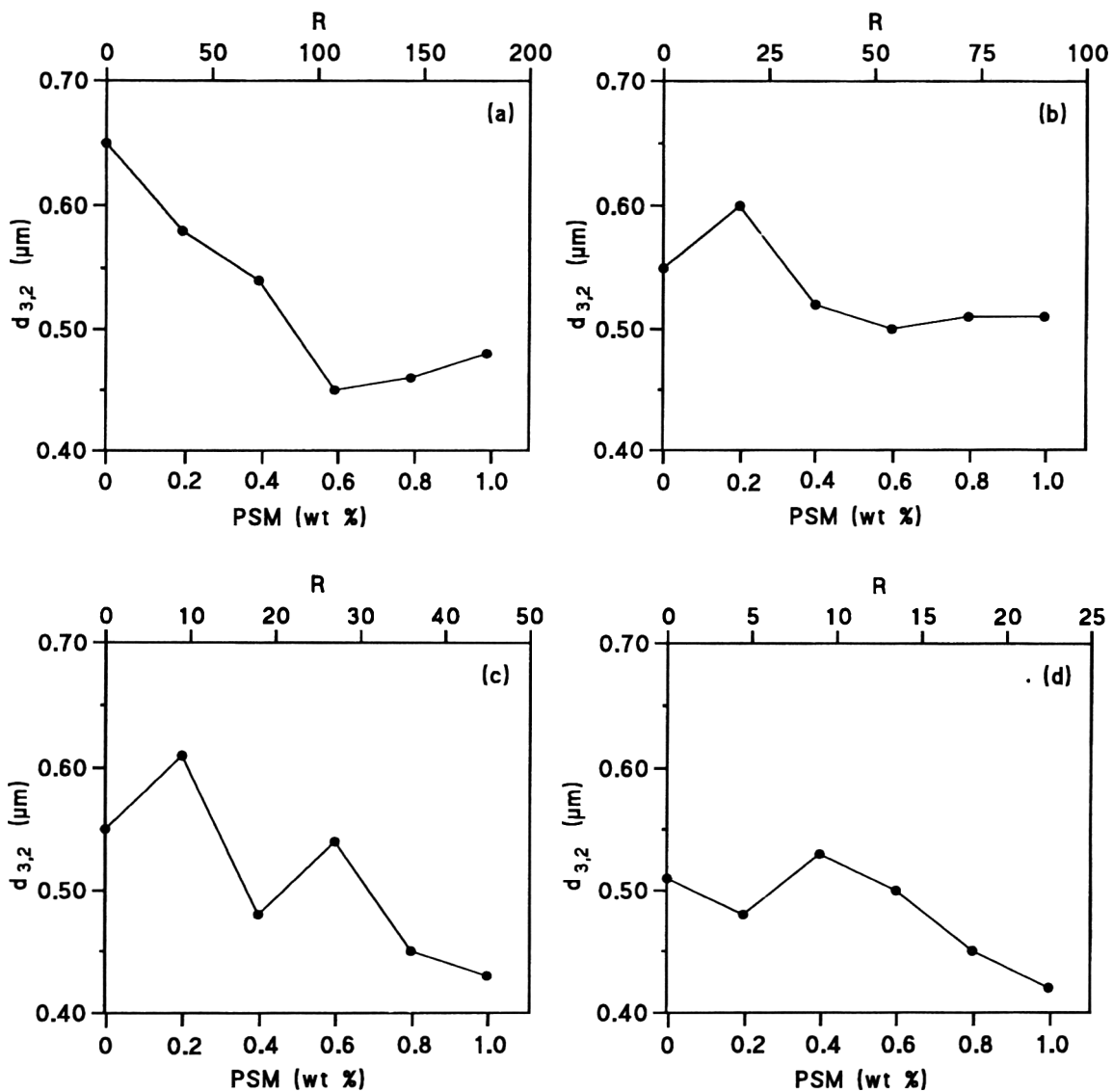
Fig. 1—Average droplet diameter $d_{3,2}$ of oil-in-water emulsions (20 wt% soya oil, deionized water, pH 7) containing various amounts of sodium caseinate and PSM. Average droplet diameter $d_{3,2}$ is plotted as a function of both the concentration of added PSM and the emulsifier-to-protein molar ratio R: (a) 0.25 wt% sodium caseinate; (b) 0.5 wt% sodium caseinate; (c) 1.0 wt% sodium caseinate; (d) 2.0 wt% sodium caseinate.

to dry. Known amounts of the dried samples were dispersed in electrophoresis buffer (pH 6.8), containing 5% SDS and 0.05% mercaptoethanol, transferred to a boiling water bath and stirred vigorously for 5 min. Aliquots of each sample were analysed by SDS-PAGE according to manufacturer's instructions (Bio-Rad Laboratories, Richmond, Ca 94804, U.S.A.), as described by Singh and Creamer (1991). The separating gels contained 15% acrylamide, made up in Tris/HCl buffer, pH 8.8, and stacking gels were composed of 4% acrylamide in Tris/HCl buffer, pH 6.8. After destaining, the integrated intensities of casein bands were determined using an Ultrascan XL model laser densitometer (LKB Produkter AB, Bromma, Sweden). The percentage composition of each casein was determined by summing the areas for α_s-, β- and κ-caseins, and expressing individual casein peaks as a fraction of the sum.

RESULTS & DISCUSSION

Water-soluble surfactant (PSM)

The volume-surface average droplet diameters $(d_{3,2})$ were compared for emulsions containing sodium caseinate and PSM (Fig. 1 a-d). The average droplet diameter $(d_{3,2})$ are plotted as a function of both the emulsifer-to-protein molar ratio (R) and the amount of added PSM. The molar ratio was calculated assuming the molecular weights of PSM and sodium caseinate were 519 and 23,000 daltons, respectively. Average droplet diameters were

in the range 0.42–0 65 μm, depending on concentration of protein and surfactant. In the absence of PSM, the average droplet diameter decreased with increasing sodium caseinate. For example, emulsions prepared with 0.25 wt% and 2.0 wt% sodium caseinate had $d_{3,2}$ values of 0.65 and 0.51 μm, respectively. In general, the presence of water-soluble surfactant during homogenization led to a reduction in emulsion droplet diameter. This was also reported by de Feijter et al. (1987), Courthaudon et al. (1991c) and Dickinson (1991). The reduction in droplet diameter was expected in view of the much greater reduction in interfacial tension in the presence of surfactant (Dickinson et al.. 1989a), and the increasing amount of surfactant material present during homogenization. There was, however, some evidence that the presence of emulsifier, at some lower R values studied in this work caused droplets to stabilize at larger sizes. This may indicate that the protein and surfactant are slightly antagonistic. rather than synergistic at those ratios, or that there was slight flocculation after the emulsion was formed.

Changes in the total amount of protein adsorbed when PSM was present during emulsification were compared (Fig. 2). In the absence of surfactant, Γ (mg/m²) increased as concentration of sodium caseinate increased. At caseinate concentrations of 0.25, 0.5, 1.0, and 2.0 wt%, the corresponding Γ values were
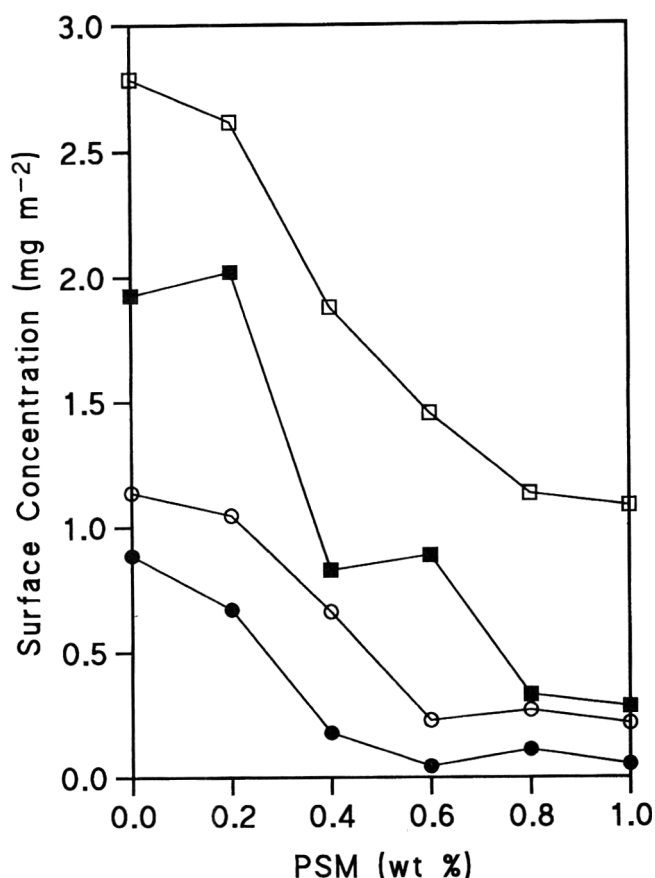
Fig. 2—Effect of PSM on the protein surface concentration Γ (mg m⁻²) in oil-in-water emulsions (20 wt% soya oil, deionized water, pH 7) containing various concentrations of sodium caseinate. The surface concentration Γ is plotted as a function of the concentration of added PSM: ● 0.25 wt% sodium caseinate; ○ 0.5 wt% sodium caseinate; ■ 1.0 wt% sodium caseinate; □ 2.0 wt% sodium caseinate.

Fig. 3—Composite plot of surface concentration Γ (mg m⁻²) against PSM-to-protein molar ratio (R) in all of the emulsions: ● 0.25 wt% sodium caseinate; ○ 0.5 wt% sodium caseinate; ■ 1.0 wt% sodium caseinate; and ⊏ 2 wt% sodium caseinate.

0.89, 1.14, 1.93, and 2.79 mg/m², respectively. This trend has also been reported by Fang and Dalgleish (1993a) and Hunt and Dalgleish (1994), in soya oil-in-water emulsions stabilized with sodium caseinate. The presence of PSM during homogenization caused a reduction in Γ at all protein concentrations. However, only partial protein displacement was observed at all concentrations of added PSM. Even at high R values (R > 100) the limiting surface protein concentration was 0.05 mg m⁻². Oortwijn and Walstra (1979) also reported incomplete protein displacement in whey protein and skim milk stabilized oil-in-water emulsions by the water-soluble surfactant, Tween 20 (Polyoxyethyene sorbitan monolaurate) at added concentrations up to 0.5 wt%. This contrasts with results reported with emulsion systems containing pure milk proteins, where water-soluble surfactant completely displaced protein from the oil-water interface. For example, Chen and Dickinson (1993) found that β-casein and β-lactoglobulin were completely removed from the droplet surface by polyoxyethylene sorbitan monolaurate at surfactant-to-protein molar ratios of R = 20 and R = 15, respectively.

The surface protein concentration (Γ mg/m²) was also plotted (Fig. 3) as a function of the emulsifier-to-protein molar ratio (R). The effect on surface concentration seemed to depend only on R, and not on the overall concentration of protein. All data points were on a single line, apart from the emulsions prepared in the absence of PSM. Previous work (Fang and Dalgleish, 1993a), has shown that the casein adopted different conformations (or spread to different extents), when different concentrations of casein were used to prepare emulsions. A possible explanation for the results (Fig. 3), is that the presence of PSM simply caused the adsorbed caseins to take a standard conformation. Thus at 2% protein, where casein binding is maximal
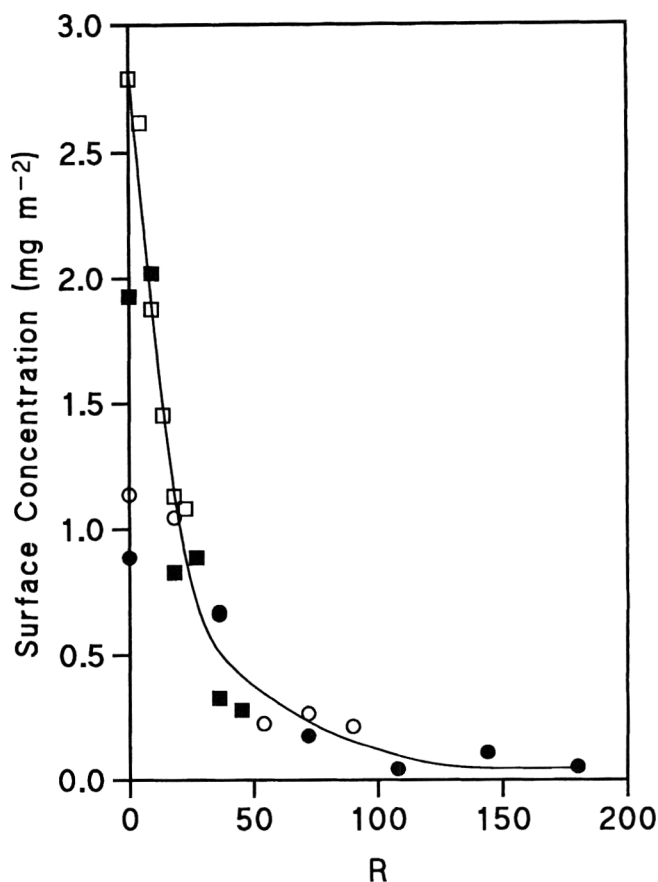
and the casein is in a conformation protruding into the aqueous phase, the protein may simply be displaced by emulsifier from the interface. At the lowest concentration of protein, where spreading would be maximal, the presence of PSM does not only displace some caseins, but allows the proteins to take a more compact conformation on the interface, so that they would cover less area, hence the increasing distance of the zero-emulsifier point from the average curve (Fig. 3). The major trend was predicted by simple Langmuir adsorption of two molecules competing for an interface:

$$\Theta_p = \frac{K_p [P]}{1 + K_p [P] + K_T [T]} \quad [1]$$

where Θ_p is the fraction of surface covered by protein, K_p and K_T are equilibrium binding constants for protein and surfactant respectively, and [P] and [T] are concentrations of protein and surfactant.

The area of interface in the emulsions was relatively fixed in our experiments (between 2 and 3 m²/g emulsion), and it seems therefore that the results of adsorption resembled those which would occur on a planar interface, even though the emulsion droplets were formed in a highly dynamic turbulent situation. This contrasts with the behaviour of emulsions formed using oil, casein and lecithin, where lecithin alters the conformation of the casein without apparently competing and causing desorption of the protein (Fang and Dalgleish, 1993b).

The composition of protein was compared at the emulsion droplet surface (Fig. 4 a–d). The proportions of α_s- and β-caseins at the emulsion droplet surface were affected by both the emulsifier concentration and the total amount of protein. However, the proportion of κ-casein at the droplet surface remained approximately the same at all added concentrations of protein
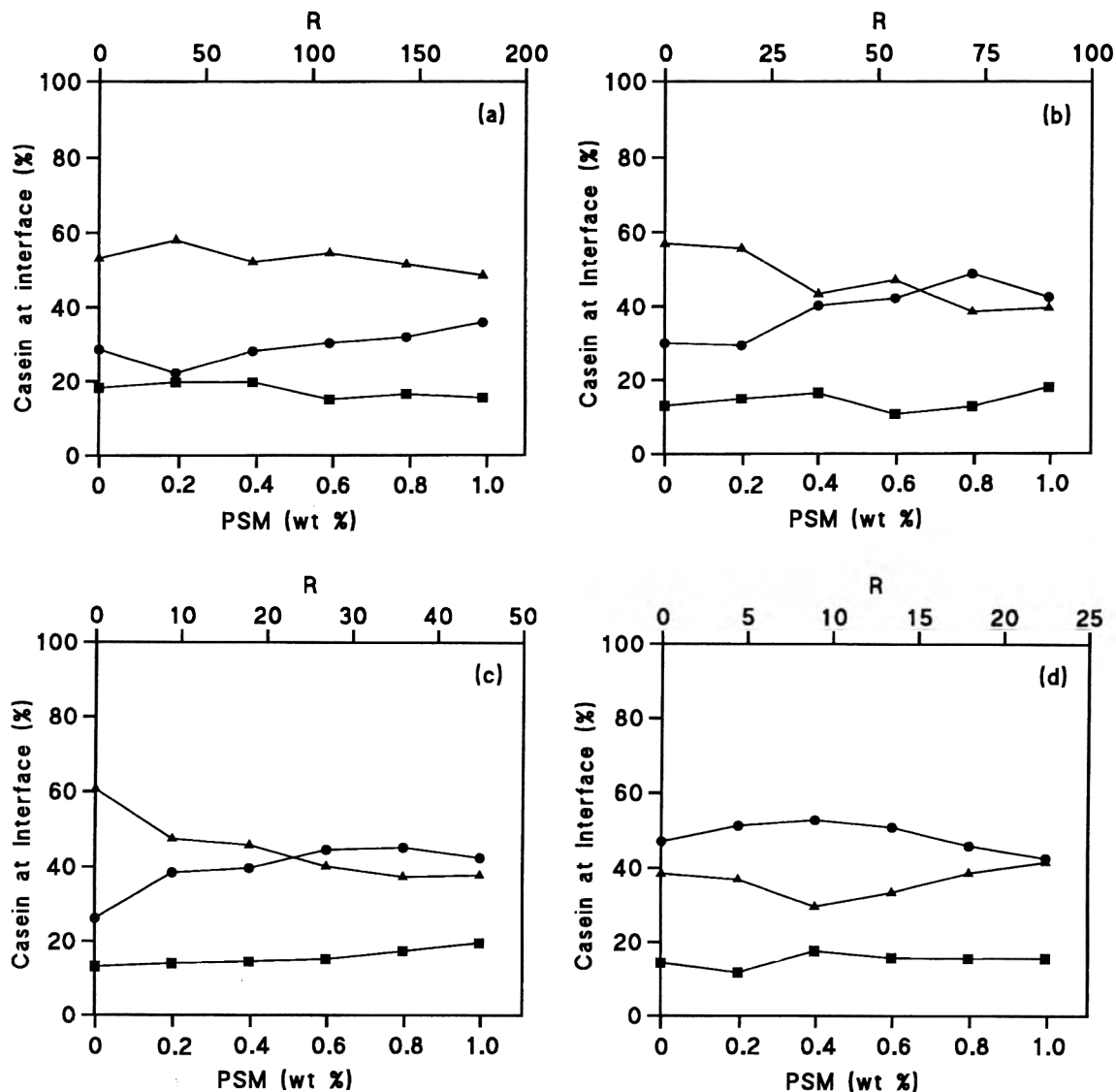
Fig. 4—Effect of PSM on the interfacial composition of caseins in oil-in-water emulsions (20 wt% soya oil, deionized water, pH 7). The proportion of each casein at the droplet surface (as % of total casein) is plotted as a function of both the concentration of added PSM and the emulsifier-to-protein molar ratio R: • α_s-casein; ▲ β-casein; ■ κ-casein; (a) 0.25 wt% sodium caseinate; (b) 0.5 wt% sodium caseinate; (c) 1.0 wt% sodium caseinate; (d) 2.0 wt% sodium caseinate.

and surfactant. In the absence of PSM, at sodium caseinate concentrations of ≤ 1.0 wt%, the ratio of β- to α_s-casein at the surface was greater than the ratio in the original sodium caseinate, suggesting that β-casein may have adsorbed in preference to α_s-casein under these conditions. However, at a protein concentration of 2.0 wt%, there was no preference for β-casein adsorption at the surface. Predominance of β-casein at the emulsion droplet surface has also been reported by Dickinson et al. (1988) in fresh emulsions (10 wt% n-tetradecane, 0.5 wt% total protein, pH 7), prepared with a mixture of pure α_{s1}- and β-caseins. This was attributed to the higher surface activity of β-casein as compared to other caseins (Benjamins et al., 1975; Dickinson et al., 1985; Castle et al., 1987). However, Robson and Dalgleish (1987) reported no preference for any of the caseins immediately after homogenization in butter oil-in-water emulsions stabilized by commercial sodium caseinate; emulsions were prepared with 1.0 wt% - 3.0 wt% protein. Only on ageing of these emulsions did β-casein displace some but not all of the surface α_{s1}-casein. In a more recent study, Hunt and Dalgleish (1994) prepared soya oil-in-water emulsions, stabilized by laboratory prepared freeze-dried sodium caseinate, using a microfluidizer. No preference for any of the caseins was found either immediately after emulsification or after ageing the emulsions. We could not explain why the results of our study differed from

that previous work. Different emulsification equipment and conditions used as well as the different types of caseinate (commercial or laboratory prepared) and analytical methods may account for differences in competitive adsorption.

It also was not clear why the preferential adsorption of β-casein was influenced by initial protein concentration. Pepper and Farrell (1982), using gel permeation chromatography, observed that increasing protein concentration in the range 0.1 to 2.94 wt% caused formation of casein oligomers in soluble whole casein. The formation of such oligomers at protein concentrations ≥ 1.0 wt% possibly affects the adsorption behavior of different casein components. Another possibility is that higher concentrations of protein favour the formation of β-casein micelles (Swaisgood, 1992), which are not adsorbed as effectively as the monomeric β-casein.

At the lowest caseinate concentration (0.25 wt%), addition of PSM caused a slight decrease in proportion of β-casein and a concomitant increase in α_s-casein at the droplet surface. At protein concentrations 0.5 and 1.0 wt%, the differences in droplet surface composition in the presence and absence of emulsifier were greater. At added emulsifier concentrations of 0.4 wt% and above, the β- and α_s-caseins were adsorbed at the surface in approximately equal amounts. This indicated more specific displacement of β-casein by PSM under these conditions. The
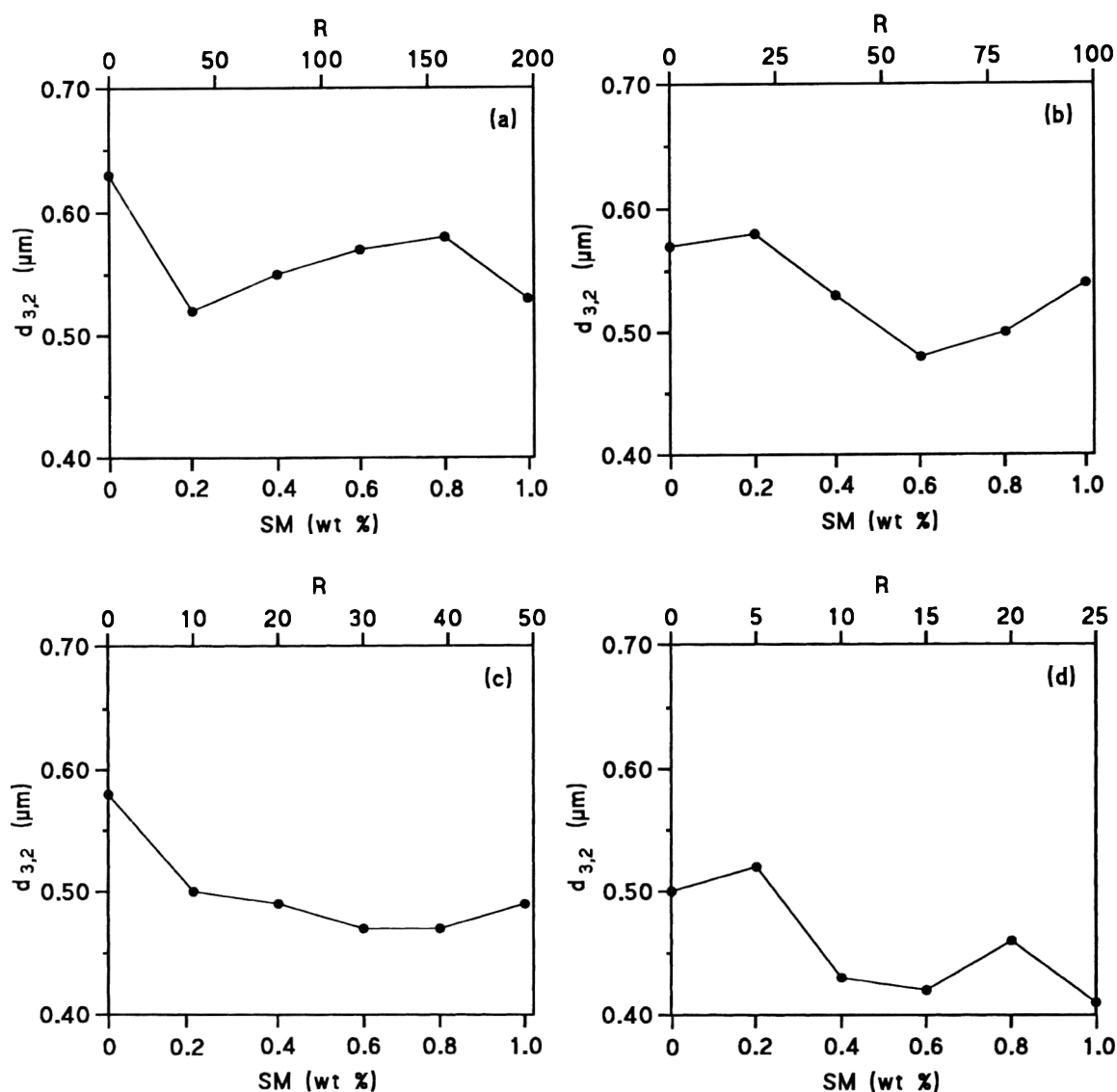
Fig. 5— Average droplet diameter $d_{3,2}$ of oil-in-water emulsions (20 wt% soya oil, deionized water, pH 7) containing various amounts of sodium caseinate and SM. Average droplet diameter $d_{3,2}$ is plotted as a function of both the concentration of added SM and the emulsifier-to-protein molar ratio R: (a) 0.25 wt% sodium caseinate; (b) 0.5 wt% sodium caseinate; (c) 1.0 wt% sodium caseinate; (d) 2.0 wt% sodium caseinate.

much greater surface activity of the β-casein molecule may enable it to interact more with PSM compared to the other two casein fractions (α_s- and κ-caseins). At a caseinate concentration of 2.0 wt%, the casein composition at the surface remained approximately the same regardless of added PSM. However, the preferential adsorption of β-casein was a relatively small effect within the general decrease; the major effect of PSM was to cause non-specific desorption of all caseins, with a slight bias to preferentially removing β-casein.

Oil-soluble surfactant (SM)

As with PSM, the presence of SM during homogenization resulted in a decrease in droplet diameter ($d_{3,2}$) but only up to emulsifier-to-protein molar ratios of $\sim$ R = 40 to $\sim$ R = 60 (Fig. 5). The molar ratio was calculated on the assumption that molecular weights of SM and sodium caseinate were 475 and 23,000 daltons, respectively. Several other workers have also observed a reduction in emulsion droplet size on addition of oil-soluble surfactant including Dickinson et al. (1989b), Courthaudon et al. (1991d) and Dickinson et al. (1993). A minimum in droplet diameter was observed at molar ratios of R = 40 to R = 60 in our emulsion systems followed by a subsequent increase in $d_{3,2}$ values. For example, in emulsions stabilized by 0.5 wt%

caseinate (Fig. 5b), the droplet diameter decreased from 0.57 μm in the absence of SM to 0.48 μm at a molar ratio of R = 60. This was followed by an increase in $d_{3,2}$ to 0.54 μm at R = 100. Similar results have been reported by Dickinson et al. (1993) who observed an increase in diameter of β-lactoglobulin-stabilized emulsion droplets on the addition of Span 80 (sorbitan monooleate) at molar ratios of R ≥ 50. This has been attributed to incipient destabilization of the oil-water emulsion by the lipophilic emulsifier.

Surface protein concentration (Γ mg/m²) was plotted as a function of concentration of added SM (Fig. 6). There was incomplete protein displacement from the droplet surface by the oil-soluble surfactant. Partial protein displacement by oil-soluble surfactant (in particular monoglycerides and phospholipids) has been well documented (Oortwijn and Walstra, 1979; Dickinson et al., 1989b, 1993; Heertje et al., 1990; Courthaudon et al., 1991d; Krog, 1991). Comparison of data shown (Fig. 6 and Fig. 2) indicate that the oil-soluble surfactant, SM, was not as effective as the water-soluble surfactant, PSM, in displacing protein from the droplet surface. Oortwijn and Walstra (1979) and Courthaudon et al. (1991a) also reported a more pronounced displacement of milk protein by water-soluble surfactants as compared to oil-soluble surfactants.
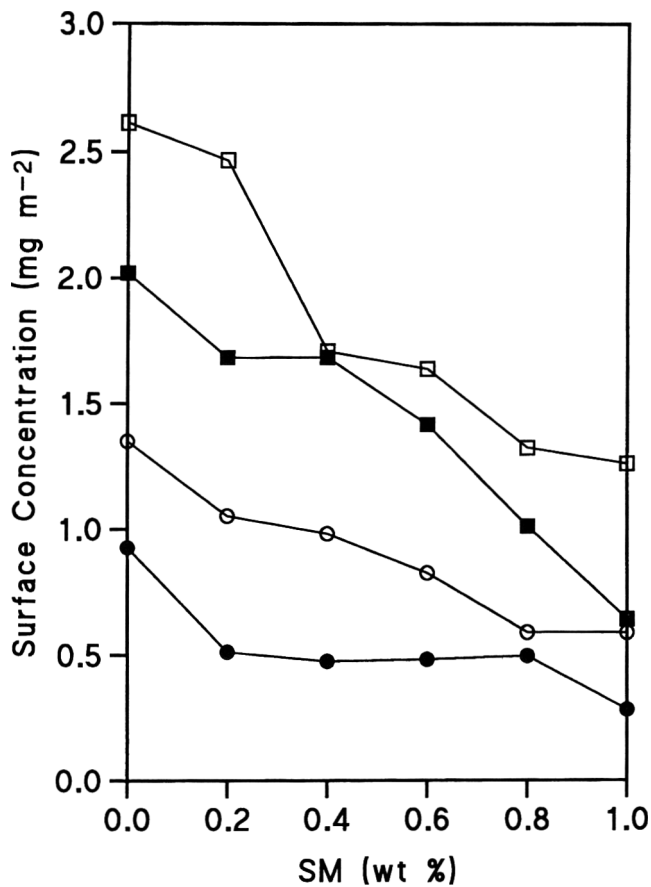
Fig. 6—Effect of SM on the protein surface concentration Γ (mg m^{-2}) in oil-in-water emulsions (20 wt% soya oil, deionized water, pH 7) containing various concentrations of sodium caseinate. The surface concentration is plotted as a function of the concentration of added SM: ● 0.25 wt% sodium caseinate; ○ 0.5 wt% sodium caseinate; ■ 1.0 wt% sodium caseinate; □ 2.0 wt% sodium caseinate.
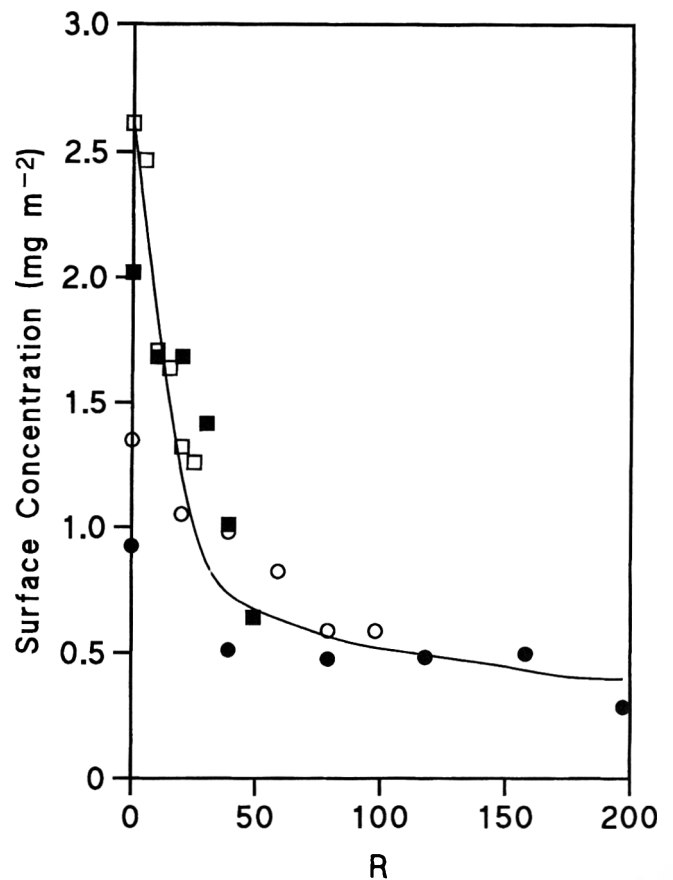
Fig. 7—Composite plot of surface concentration Γ (mg m^{-2}) against SM-to-protein molar ratio (R) in all of the emulsions: ● 0.25 wt% sodium caseinate; ○ 0.5 wt% sodium caseinate; ■ 1.0 wt% sodium caseinate; and □ 2.0 wt% sodium caseinate.

As with PSM (Fig. 3), we could combine results from all emulsions in one figure (Fig. 7). This once again suggested that the emulsions made with protein alone were different in their behaviour, because of the spreading of the caseinate. Comparison of results (Fig. 3 and Fig. 7) shows that SM had less influence on configuration of protein than PSM at the lower protein concentrations. The combined curve shows graphically that there was virtually no ratio of SM to protein large enough to result in complete displacement of the protein.

Proportions of caseins at the emulsion droplet surface were plotted as a function of the amounts of added SM at different protein concentrations (Fig. 8 a–d). Trends were quite similar to those observed for PSM (Fig 4. a–d). In the absence of surfactant, preferential adsorption of β-casein at the droplet surface occurred in emulsions containing ≤ 1.0 wt% sodium caseinate. At the highest concentration of protein used in this experiment (2.0 wt%) the caseins were adsorbed at the droplet surface according to their proportions in the bulk phase. The addition of SM (Fig. 8d) did not alter the proportions of individual caseins adsorbed at the emulsion droplet surface. In emulsions containing 0.25 or 0.5 wt% sodium caseinate and ≤ 0.6 wt% added SM, there was a marked preference for β-casein at the droplet surface. This preferential adsorption of β-casein was lessened, with a corresponding increase in proportion of α_s-casein at the interface with added SM concentrations of ≥ 0.8 wt%. SM had the most effect on the interfacial protein composition in emulsions containing 1.0 wt% sodium caseinate (Fig. 8c). As the amount of SM was increased in these systems, the preference for β-casein for the droplet surface was substantially reduced. There

was no preference for β-casein at the interface in emulsions containing added concentrations of ≥ 0.8 wt% SM.

One important application of low-molecular weight surfactants is the weakening of protein-fat binding to cause agglomeration of fat globules in whippable emulsions such as ice cream mix or whipped cream (Berger, 1976; Darling and Birkett, 1987). This leads to products with high melt resistance and a smooth texture (Goff and Jordan, 1989). Our results suggest that the water-soluble surfactant PSM, which can cause greater protein displacement, should cause more fat destabilization in such systems than SM. Goff and Jordan (1989) demonstrated that PSM produced significantly more fat destabilization in standard ice cream mix than did SM.

Research is needed on the competitive adsorption between sodium caseinate and a mixture of surfactants, since most food emulsions involve a combination of emulsifiers. It would be of special interest to study PSM, SM and mono- and diglycerides, since these surfactants are commonly used in food emulsions.

CONCLUSIONS

BOTH THE WATER-SOLUBLE SURFACTANT, PSM and the oil-soluble surfactant, SM could partially displace sodium caseinate from the oil-water emulsion interface. Of the two surfactants, PSM was the more effective in reducing protein surface concentration. The composition of casein at the droplet surface was independent of surfactant type. The most surface-active casein (β-casein) predominated the interface in the absence of surfactant in emulsions containing ≤ 1.0 wt% sodium caseinate. The preference for β-casein at the droplet surface was substantially reduced in the presence of surfactant, especially in emulsions stabilized by 0.5 and 1.0 wt% sodium caseinate. At an added
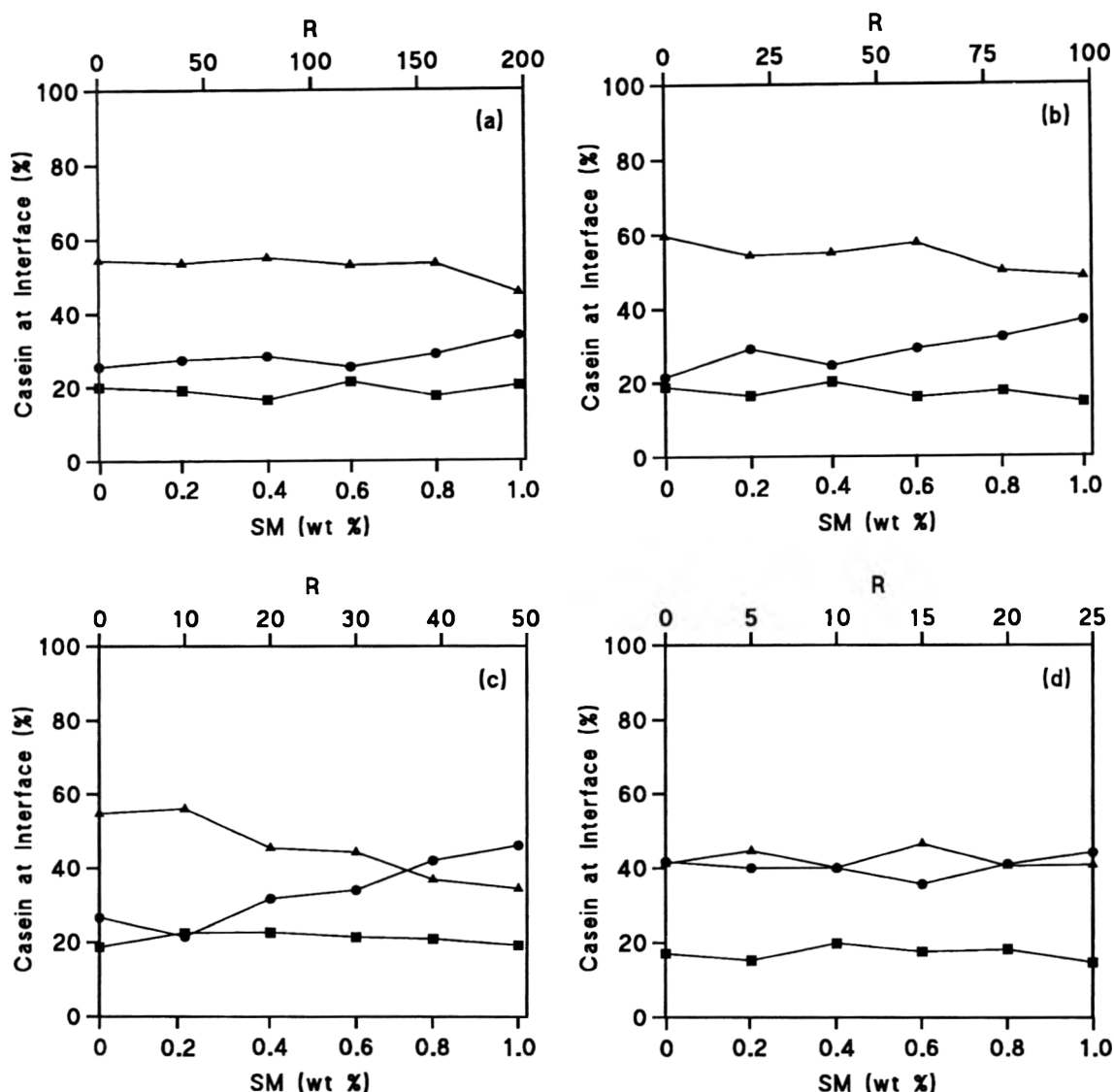
Fig. 8—Effect of SM on the interfacial composition of caseins in oil-in-water emulsions (20 wt% soya oil, deionized water, pH 7). The proportion of each casein at the emulsion droplet surface (as % of total casein) is plotted as a function of both the concentration of added SM and the emulsifier-to-protein molar ratio R: ● α_s-casein; ▲ β-casein; ■ κ-casein; (a) 0.25 wt% sodium caseinate; (b) 0.5 wt% sodium caseinate; (c) 1.0 wt% sodium caseinate; (d) 2.0 wt% sodium caseinate.

sodium caseinate concentration of 2.0 wt%, preferential adsorption of β-casein was not observed either in the presence or absence of surfactant.

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HEAT STABILITY OF OIL-IN-WATER EMULSIONS. . .

mation during heating at high ionic strength. This behavior illustrated the importance of the composition of free protein in the emulsion with respect to heat stability and also confirmed the function of emulsion droplets in facilitating gel formation.

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Power Absorption During Microwave Heating of Emulsions and Layered Systems

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——————— ABSTRACT ———————

Microwave oven heating rates of various oil-in-water emulsions, water-in-oil emulsions, and layered systems were compared. Emulsions heated faster than corresponding layered systems, which heated faster than a calculated weighted average of individual components. Differences were attributed to increased power absorption caused by the large number of interfaces occurring in emulsions, or by a single interface in the layered system, and by resonant absorption of microwave radiation, which is determined by dielectric properties. The types and proportions of emulsions determined the number of interfaces. The dielectric properties and sample size determined in which samples resonant absorption occurred.

Key Words: emulsions, oil-water, layered, heat transfer, microwave

INTRODUCTION

THE MICROWAVE OVEN has many commercial and consumer uses, especially for heating foods. An emulsion is a common food system that forms the basis of salad dressings, cakes, sausages and many other foods. Understanding how an emulsion heats in a microwave oven can be of advantage because of the wide usage of microwave systems.

The heating rate of a multicomponent system, such as an emulsion, has not been thoroughly studied in the microwave oven. From knowledge of conventional heating, we may predict that the heating rate of an emulsion would equal the heating rate of the weighted average of oil and water. That is, the heat capacity would be the major determinant. However, the fundamental nature of energy absorption by the sample in the microwave oven is different from that in the conventional oven. Maxwell's field equations (Maxwell, 1881) predict that at every interface, part of the microwave radiation is reflected. An emulsion consists of droplets dispersed in a continuous phase, with an interface around every droplet. Therefore, the absorption of power should be different for an emulsion, which has many interfaces, than for pure oil or water, which has only an external interface. Sasaki et al. (1988) reported the heating rates of oil, water and emulsions in a microwave oven. They concluded that the power absorption of the entire system equaled the power absorbed by the water plus that absorbed due to the interfacial area, which could be closely approximated by measuring only the power absorption of the water phase. At that time they did not have an explanation as to why an interface increased power absorption.

Whether the continuous phase is oil or water will also affect an emulsion's heating rate. The electrostatic solution for the electric field inside a drop (Stratton, 1941) indicates that the electric field depends on which component is the continuous phase. In an oil-in-water emulsion, the electric field inside the oil droplet would be enhanced over the field in the continuous water phase. In the reverse emulsion, water-in-oil, the field inside the water droplet would be reduced over the field in the continuous oil phase. Because of this difference in electric field, the two emulsions should have different dielectric values. Thomas et al. (1990) found this was true for emulsions in the gigahertz range, but they did not go as low as 2450 MHz, the frequency of home microwave ovens.

Previous work (Barringer et al., 1994) on heating rates of oil and water indicates that for samples with given dielectric properties, sample size determines which heat transfer mechanisms dominate during heating. They found that for samples in which the beaker radius was large compared to the wavelength of radiation within the sample, heating rate was controlled by thermal properties, especially heat capacity. For samples on the order of, or smaller than, that wavelength, the dominant effects were the reflections and wave interferences that occur as electromagnetic radiation interacted with the sample.

In our investigation, emulsions were heated in the microwave oven. Most experiments were done with 20-g samples so that electromagnetic interactions would be dominant. Oil-layered-on-water samples were also heated in the microwave oven. Our objective was to study the underlying mechanism for the heating rate by varying the proportions of oil to water and type of emulsion, in comparison to a layered system.

MATERIALS & METHODS

OIL-IN-WATER (o/w) and water-in-oil (w/o) emulsions were prepared with various amounts of water. Xanthan gum and emulsifiers were added as stabilizers. Xanthan gum (Keltrol T, Kelco Division of Merck and Co. Inc., Rahway NJ) was dissolved in three times its weight of oil (Mazola, Best Foods, Englewood Cliffs, NJ) and then added at a 1% level to distilled water. The solution was mixed for 5 min with a hand mixer (model 4166, Braun, Lynnfield, MA) to hydrate the gum. The emulsifier was dissolved in the oil. For o/w emulsions, polysorbate (Polycon T60, Humko Chemical Division of Witco Corporation, Memphis TN) was used at 0.2% of the oil, the level recommended for o/w salad dressings. For w/o emulsions, saturated monoglyceride (Dimodan PVK, Grindsted Products Inc, Industrial Airport, Kansas) was used at 1% of the oil, the minimum needed to form a stable emulsion.

The oil-emulsifier and water-gum phases (hereafter referred to as the oil, and water, phases) were mixed 2 min with the hand mixer. The mixer was set on the highest setting to produce a medium emulsion and the lowest setting to produce a coarse emulsion. The final emulsion was degassed for 30 min under 25 mmHg vacuum and held at 4°C. To determine emulsion droplet size, samples were observed under a microscope. Emulsions were classified as coarse if droplets had a diameter of ≤ 200 μm and medium if all droplet diameters were < 10 μm.

A second set of emulsions was prepared with 1% NaCl added to the water phase. A third set was prepared by layering the oil phase on top of the water phase without mixing.

Samples (emulsion, water or oil) were placed in beakers and refrigerated to 4°C. For 50% oil 50% water samples, 20, 30, 50, 100, 200, 300, 400, 500, 600, and 700g samples were used. They were then placed in the center of a household microwave oven (Model 1285, Litton, Minneapolis, MN) with a measured output of 600W. Samples were heated to an end temperature of 55°C. The time required to reach this end temperature was determined for each sample. At the end of heating, samples were stirred 30 sec to determine the final temperature. From the

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initial and final temperatures, the average heating rate was determined. Initial and final temperatures were measured with a thermocouple. The average and standard deviation of heating rates was plotted, though in many cases standard deviations were very small.

The dielectric properties were measured using a dielectric probe (model 85070A Hewlett-Packard Co., Santa Rosa, CA). The specific heat, thermal conductivity, and density were calculated based on a weighted average of water and oil. The values for water and oil were those of Earle (1973.) The heat transfer coefficient was measured experimentally for still air.

THEORY

When microwave radiation reaches an interface between two media with different dielectric properties, a fraction of the energy is reflected, with the remainder transmitted into the second media. The percent transmitted power at an interface, or the ratio of transmitted power, P_{trans}, to the incident power, P_{inc}, can be calculated from the transmission coefficient between medium 1 and medium 2, $T_{1,2}$, and the wavelength within the medium, λ_s, for medium 1 and medium 2:

$$\frac{P_{trans}}{P_{inc}} = (T_{1,2})^2 \frac{\lambda_{s1}}{\lambda_{s2}} \quad (1)$$

where $T_{1,2}$ is

$$T_{1,2} = \sqrt{\frac{4(\alpha_1^2 + \beta_1^2)}{(\alpha_1 + \alpha_2)^2 + (\beta_1 + \beta_2)^2}} \quad (2)$$

α, the phase factor is:

$$\alpha = \frac{2\pi}{\lambda_s} \quad (3)$$

and β, the attenuation factor is:

$$\beta = \frac{\pi\sqrt{2\kappa'}\left(\sqrt{\sqrt{1 + \left(\frac{\kappa''}{\kappa'}\right)^2} - 1}\right)}{\lambda_0} \quad (4)$$

where λ_0 is the wavelength in free space, or 12.24 cm at 2450 MHz. The wavelength within the sample, λ_s, is determined from the dielectric properties, κ' and κ'':

$$\lambda_s = \frac{\lambda_0}{\sqrt{\frac{\kappa'}{2}}\sqrt{\sqrt{1 + \left(\frac{\kappa''}{\kappa'}\right)^2} + 1}} \quad (5)$$

The dielectric properties are the real portion, κ', and imaginary portion, κ'', of the relative complex permittivity, κ^*.

The electric field inside a dispersed droplet, E_i, can be calculated from (Stratton, 1941):

$$E_i = \frac{3 E_o}{\left(\frac{\kappa'_{drop}}{\kappa'_{bulk}} + 2\right)} \quad (6)$$

where E_o is the field outside the drop, κ'_{drop} the dielectric constant of the discontinuous phase and κ'_{bulk} the dielectric constant of the continuous phase. Calculated for our system, in an o/w emulsion the field inside a droplet is $1.477E_o$ while for a w/o emulsion the field inside a droplet is $0.089E_o$. This accounts for the difference seen in the measured dielectric properties for the two types of emulsions.

RESULTS & DISCUSSION

MEASURED AVERAGE HEATING RATES were compared (Fig. 1) for the oil-in-water emulsions, water-in-oil emulsions and layered system with and without added NaCl. Results for the me-

dium emulsions are presented, as the heating rates for coarse and medium emulsions were similar. Emulsions that had concentrations of dispersed phase greater than those shown were not stable and could not be tested.

Oil-in-water emulsions, no NaCl added

The average heating rates for oil-in-water emulsions increased as concentration of dispersed phase (oil) increased (Fig. 1a.) In conventional heat transfer, the average heating rate for a sample can be estimated by calculating the weighted average of individual components, assuming no interactions. Therefore, the heating rate of the two phases: water and oil; were measured separately. The heating rate for an emulsion calculated as the weighted average of these two components is shown as the dotted line (Fig. 1a.) If these emulsions were being heated conventionally, their average heating rates would be expected to fall along this line. However, the heating rate for emulsions in the microwave oven was much higher than this prediction (Fig. 1a). The heating rate of the emulsion varied from 3°C/sec to almost 6°C/sec while the weighted average decreased from 3°C/sec, for the water phase alone, to <1°C/sec over the same concentration range.

An alternative theory to predict the heating rate of an emulsion was proposed by Sasaki et al. (1988) based on studies with 100, 200, and 500-g samples. They suggested that the power absorption of an emulsion is approximately the same as an equivalent volume of water. Thus the heating rate of emulsions would be the same as the heating rate of water. While this gives a closer prediction than conventional heating theory, it predicts a heating rate of 3°C/sec while the emulsions were actually heating at 3 to 6°C/sec. Neither of these predictions were accurate for 20-g samples in the microwave oven (Fig. 1a). At all water concentrations, the oil-in-water emulsion heated faster than either the weighted average of oil and water heating rates or pure water.

For the most concentrated emulsion that was stable, 80% oil in 20% water, the measured heating rate was 4.7 times the rate predicted from conventional heating (Fig. 1a). Also, the emulsion heated faster than the oil layered on top of the water system of the same composition (Fig. 1c). Thus, predictions for the heating rate of an emulsion in the microwave oven based on its components without considering the configuration of the components could greatly underestimate the rate.

The reason for this is that the heating rate was affected by two variables for these samples. The convective heat transfer between the sample and surrounding air was negligible in these experiments, so the power absorption could be expressed as $P = mC_p\rho$ ($\Delta T/\Delta t$). The mass, m, was identical for all samples. The density, ρ, was so similar for oil and water that little error is introduced if the density is assumed to be identical. The heating rate, $\Delta T/\Delta t$, was measured in the experiment. The remaining variables were the power absorption, P, and heat capacity, C_p. Heating rate differences between samples must therefore be dependent on differences in either heat capacity or power absorption.

Sample heat capacity will affect heating rate. An emulsion with a lower water content will have a lower C_p due to the larger volume of low heat capacity oil. This would result in an increase in heating rate if the power absorption was the same. However, this explains only 8.6% of the heating rate increase for the emulsions with the highest concentrations of oil, and even less for other emulsions.

The power absorption also affects the heating rate and it appears that this was the primary effect unique to microwave heating. Sasaki et al. (1988) proposed that part of the power absorption of an emulsion was due to the water-oil interfacial area. Comparing the heating rate of the emulsions (Fig. 1a) to the layered systems (Fig. 1c), samples with identical composition, but as an emulsion instead of layered, could have up to 53% increase in heating rate. Given that the composition was iden-
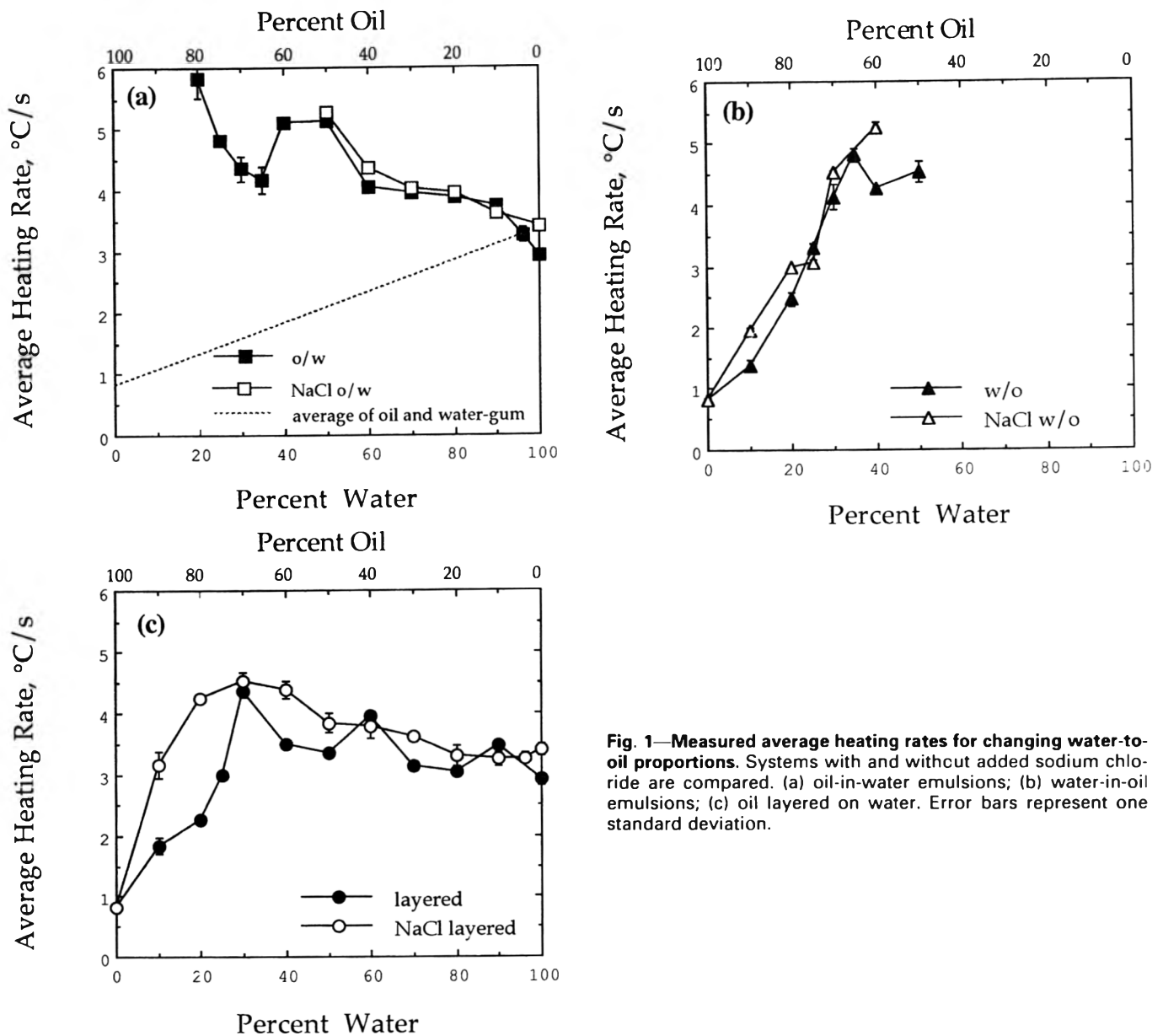
Fig. 1—**Measured average heating rates for changing water-to-oil proportions.** Systems with and without added sodium chloride are compared. (a) oil-in-water emulsions; (b) water-in-oil emulsions; (c) oil layered on water. Error bars represent one standard deviation.

tical, the increase in heating rate must have been caused by changes in conformation. Formation of an emulsion created a large number of droplets dispersed in a continuous phase. Surrounding every droplet was an interface, and at every oil-water interface, 53% of the radiation was reflected (calculated from Eq. 1). These multiple internal reflections would increase the percent of available power absorbed before the radiation exits the sample since the internal pathlength increased. This would increase the total power absorption over that for a sample where no reflections occur, given that the amount of power available was not limiting. This resulted in the observed sample heating rate being higher than expected (Fig. 1a).

Another determinant of power absorption is the sample's dielectric properties. The dielectric properties of various emulsions were measured and used to calculate the theoretical heating rate. That rate was calculated from a model using Maxwell's field equations to determine the power absorption within an infinite cylinder of homogeneous material, with the measured dielectric properties, placed in a unidirectional microwave field (Ayappa et al., 1992). The modeling was done to show the heating rate of the emulsions in relation to each other, therefore the incident power level was arbitrarily set at 45,000 W/cm². That level is so high that no one could mistakenly think that this was an attempt to predict actual heating rates. The model assumed

the emulsion to be a homogeneous material with no internal interfaces. Thus, the model eliminated the effects of internal reflections and only examined the effects of changing dielectric properties.

Barringer et al. (1994) demonstrated that for a sample with given dielectric properties, the power absorption and heating rates are oscillatory for different beaker radii. That is, a slight change in beaker radius could give a large change in heating rate. In our experiments the beaker size was held constant but samples with different dielectric properties were tested. In both situations, a resonant condition was created for certain combinations of beaker size and dielectric properties. Power absorption maxima occur where the combination of beaker radius and dielectric properties cause constructive wave interference within the sample to be at a maximum. Power absorption minima occur where destructive wave interference is at a maximum.

Theoretical peaks of power absorption occur at 20, 40 and 70% water (Fig. 2), similar to peaks shown experimentally at 20 and 40% water in the o/w heating data (Fig. 1a). Thus, the dielectric properties caused some samples to heat more rapidly, due to constructive wave interference, than would be otherwise expected. The dielectric properties and interfacial reflections combined to produce a higher heating rate for emulsions than for the corresponding layered system.
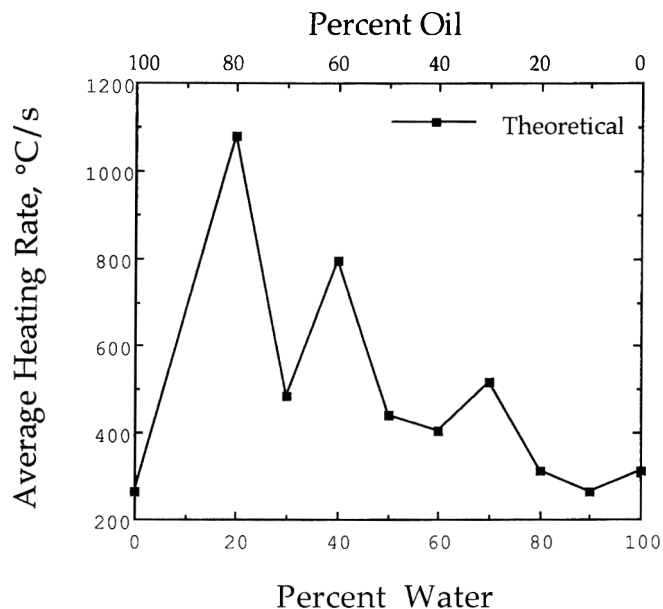
Fig. 2—Theoretical average heating rate of oil-in water emulsions for changing water-to-oil proportions.
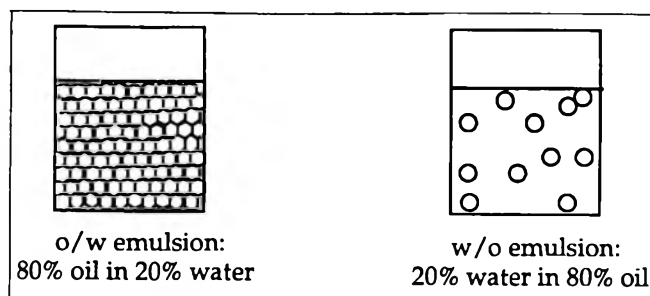

Fig. 3—Two emulsions with the same composition but different continuous phase. Packing of droplets is very different for the two cases.
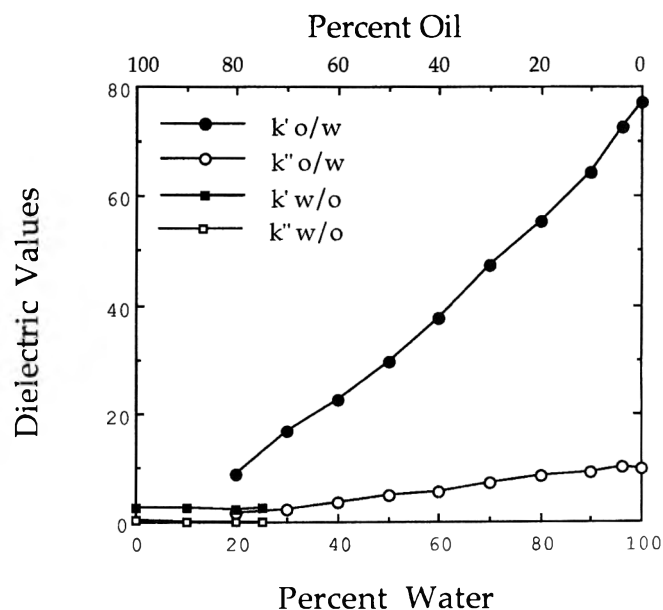

Fig. 4—Measured dielectric constant (k') and loss (k") for the oil-in-water and water-in-oil emulsions for changing water-to-oil proportions.

Water-in-oil emulsions, no NaCl added

The heating rate of the w/o emulsions (Fig. 1b) increased as percent water increased, apparently leveling off at 30% water where the emulsion destabilized. This was opposite of results with o/w (Fig. 1a), where the heating rate generally decreased with increasing water content. The data can also be interpreted as increased power absorption with an increased concentration of dispersed phase. In both types of emulsions, increased concentration of dispersed phase increased the heating rate. As the concentration of dispersed phase increased, the number of interfaces, and thus interfacial reflections, increased. This would increase power absorption and consequently heating rate.

To further illustrate the importance of the number of interfaces on heating rate, compare the two types of emulsions at 20% water (Fig. 1a and 1b). Figure 3 illustrates schematically the difference in packing for these two emulsions with identical composition, except for type of emulsifier, but different configuration. The o/w emulsion is so tightly packed that the droplets are distorted. The w/o emulsion has dispersed droplets separated by large amounts of oil. For these samples, the w/o emulsion (Fig. 1b) heated at 43% of the rate of the o/w emulsion of the same concentration (Fig. 1a). The oil to water proportions were the same for these emulsions, however the number of interfaces were different, resulting in very different heating rates. Additionally, the dielectric properties of the emulsions were different.

The importance of dielectric properties in determining the heating rate can be seen by comparing heating rates of samples with the same number of interfaces. If number of interfaces were the only effect, we would expect that a 20% water in 80% oil (w/o) emulsion would heat at the same rate as a 20% oil in 80% water (o/w) emulsion. However the o/w emulsion heated 1.5 times faster (Fig. 1a) than the w/o emulsion at the same concentration of dispersed phase (Fig. 1b). This is most likely because the dielectric values for the two samples were different (Fig. 4). The dielectric constant was 2, dielectric loss of 0.1 for w/o, while for o/w the dielectric constant was 9, dielectric loss of 2. This difference in dielectric values resulted in a difference in heating rate.

The nature of the continuous phase can also be shown to affect heating rate. Comparing two samples with identical composition and number of interfaces, 50% oil and 50% water (Fig. 1a and b), the configuration of the components still affected the sample heating rate. The o/w emulsion heated 13% faster than the w/o emulsion. Theoretically from Eq. (6), when oil is the continuous phase it shields the water droplets and reduces the electric field within the drop to 0.0753 times the field in the oil phase. When water is the continuous phase, the field within the oil drop is enhanced to 1.48 times the field in the water phase. This difference in internal electric field affects the power absorption of the sample and thus the heating rate.

Layered system, no NaCl added

The oil-in-water emulsion (Fig. 1a) had a greater heating rate than the layered system (Fig. 1c). However, a much larger increase occurred between the heating rate of the layered system (Fig. 1c) and the weighted average of individual components (dotted line, Fig. 1a). The reverse emulsion, water-in-oil (Fig. 1b), had a heating rate similar to the layered system (Fig. 1c), but still faster than the weighted average (dotted line, Fig. 1a). This demonstrated that even one interface could greatly affect the heating rate. The oil-water interface theoretically reflects 53% of the radiation, greatly increasing the power absorption within the sample. Each successive interface continued to add reflections but the effect on heating rate should become smaller as the number of interfaces increased because the power absorption could only be increased to the maximum power available. This can be shown by changing the sample size (Fig. 5).

As samples increased in size, a greater percentage of the radiation was absorbed by the sample, even if there were no internal interfaces. Therefore, the addition of interfaces should
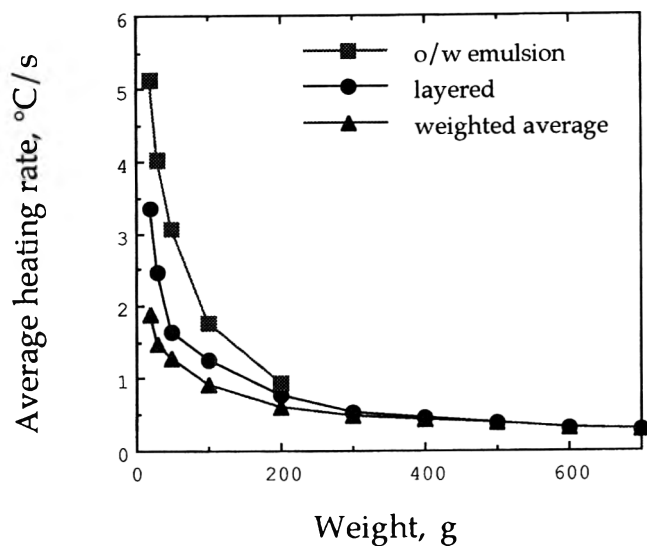
Fig. 5—Measured average heating rate for an oil-in-water emulsion, layered system and weighted average with 50-50 oil and water. Samples are compared for various sizes.
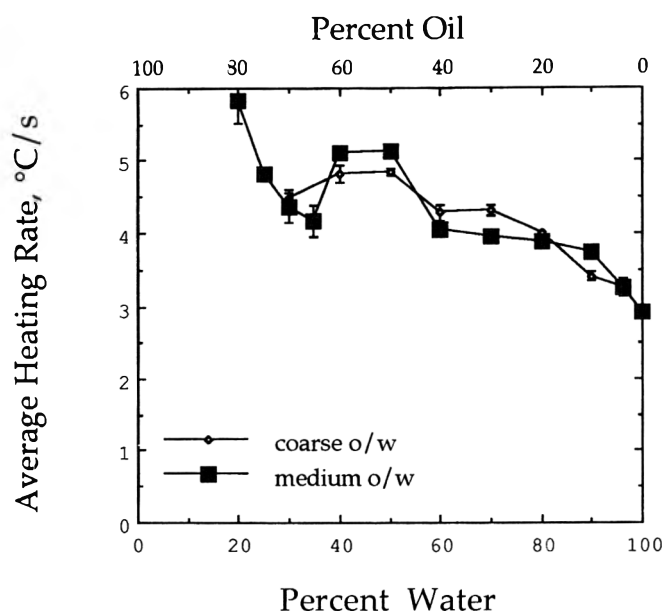

Fig. 6—Measured average heating rates of oil-in-water emulsions for changing water to oil proportions. A coarse (droplet diameter up to 200 μm) and medium (droplet diameter less than 10 μm) emulsion are compared.

have a smaller effect on the heating rate of large samples, than on the heating rate of small samples (Fig. 5). The heating rates were compared for a 50-50 oil and water sample, configured as an o/w emulsion, as a layered system, and calculated for the average of the individual components; with the total sample size ranging from 20g to 700g (Fig. 5). At the smaller sample sizes, the emulsion heated faster than the layered system, which heated faster than the weighted average. As sample sizes increased, the difference between the three samples decreased until they were all heating at a similar rate.

NaCl-water emulsions

A second set of emulsions was prepared with added NaCl. The addition of NaCl increases the dielectric properties of the

water phase, while having little effect on droplet size. Therefore, the NaCl was added to test the importance of dielectric properties as compared to interfacial reflections. NaCl-water heated faster than pure water when layered under oil (Fig. 1c). This was expected, as the addition of salts increased the dielectric loss which should increase heating rate. If the nature of the individual components were the most important factor to the heating rate, the NaCl-water emulsions would heat faster than the no-NaCl emulsions. When the NaCl-water and oil were made into an emulsion, however, the resulting heating rates were only slightly higher, or the same as the no-NaCl emulsions (Fig. 1a and b). This was true for both o/w and w/o emulsions. The elevated power absorption due to interfaces appeared to have a larger effect than individual heating rates of the components.

Droplet size

Changing the droplet size of the emulsion changed the heating rate slightly (Fig. 6). The medium emulsion had more interfaces, thus should absorb more power, but the dielectric properties were also changed which changed the wave interference effects. Also, the difference in droplet diameters between the coarse (up to 200 μm) and medium (under 10 μm) emulsion may not be large enough to show a difference. The result was that the heating rate did not vary consistently with droplet size.

CONCLUSIONS

THE HEATING RATE of a multicomponent sample in the microwave oven is related to the configuration of components as well as the nature of the components. A sample with multiple interfaces heats faster than one with only a few interfaces, due to increased power absorption from the large number of reflections at those interfaces. Therefore, without changing the formulation, the heating rate of a sample may be increased by increasing the number of interfaces. The dielectric properties may also determine heating rate by creating a resonant condition where power absorption is maximum for certain samples. Controlling sample size, relative to dielectric properties, can also increase heating rate. However, increasing the dielectric properties of one phase, such as by the addition of NaCl, may not greatly increase the overall heating rate when other factors have a larger effect. Better understanding of such effects will enable product configurations to optimize the heating rate desired.

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Ms received 9/24/94; revised 3/30/95; accepted 5/15/95.

Microwave-Heating Temperature Profiles for Thin Slabs Compared to Maxwell and Lambert Law Predictions

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—————————— ABSTRACT ——————————

Slabs of agar gel were heated in a microwave oven. Temperatures were measured at various depths into the sample to experimentally determine the internal temperature profile. These were compared to power and temperature profiles predicted from Lambert's law, Maxwell's field equations and a Combined equation. Lambert's law and the Combined equation predicted a much slower heating rate than found experimentally, while Maxwell's field equations gave a much more accurate prediction. Because of the internal standing waves that are created, a small variation in sample thickness could make a large difference in heating rate for thin samples.

Key Words: agar gel, heat penetration, Lambert's law, Maxwell's Field, microwave

INTRODUCTION

WITH INCREASING MICROWAVE OVEN use there is a need to predict the temperature profile in a microwave heated food sample. Heat generation can be modeled using either Lambert's law or the more rigorous solution of Maxwell's equations to determine the microwave power term. Both sets of equations have been reported in heating profile predictions (de Wagter, 1984; Jia and Jolly, 1992; Ohlsson and Bengtsson, 1971; Padua, 1993; Stuchly and Hamid, 1972).

Lambert's law predicts an exponentially decaying absorption of energy as a function of depth into the sample. It is valid for semi-infinite samples only since all reflections are neglected. An approximation of the percent radiation transmitted at the air-sample interface has to be determined experimentally. This is generally done by measuring the heating rate of a beaker of water and calculating the power absorbed by the sample (Ohlsson and Bengtsson, 1971; Stuchly and Hamid, 1972; Mudgett, 1986). This calorimetric determination introduces errors as it is based on the assumption that all transmitted energy is absorbed, which may not be true for thin samples.

Maxwell's equations (Maxwell, 1881) predict the absorption of energy, incorporating reflections at the front and back surfaces and all internal interfaces. Wave interactions are included, so that internal standing waves are calculated, and the electric field distribution and absorbed power are consistently evaluated. Lambert's law is a simplification of the electromagnetic interactions and is frequently used in predictive models because the calculations are much simpler than for Maxwell's field equations.

The penetration depth is defined as the depth at which the power has decayed to e^{-1} of its initial value at the front interface

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and is the inverse of Eq. (2) (Theory section). For example, the penetration depth of water is 3.6 cm. For samples thicker than several penetration depths, internal reflections become negligible because all transmitted radiation is absorbed. Ayappa et al. (1991) compared the power distributions predicted by Maxwell's equations and Lambert's law and showed that the two formulations predicted identical power profiles for samples thicker than 2.7 times the penetration depth. For thinner samples, Maxwell's equations predicted a very different profile from Lambert's law because the radiation reflected back through the sample from the far interface increased the overall power absorption. Theoretically, neglecting these back reflections for thin slabs, as happens with Lambert's law, introduces large errors (Fu and Metaxas, 1992). Experimentally, the inclusion of wave interactions was necessary to explain the heating rates of small cylinders of water (Barringer et al., 1994) and emulsions (Barringer et al., 1995).

The theoretical depth at which Lambert's and Maxwell's equations converge is 7.8 cm for agar. Few foods heated in the microwave oven are that thick. Therefore two sample thicknesses reasonable for an entree in the microwave oven were tested to determine how different the predictions were for such samples.

The objective of our research was to determine experimentally if Maxwell's field equations predicted heating rates significantly better than Lambert's law for thin samples. Measured temperatures were compared to those predicted by Lambert's law, Maxwell's equations and a Combined equation. The Combined equation was a limiting form of Lambert's law derived from Maxwell's equations. It neglects internal reflections but accounts for reflections at the incident face of the sample. The Combined equation was used to test whether a calculated transmission value would give a more accurate approximation than Lambert's law without introducing the complexities of internal reflections.

THEORY

Power from Lambert's law

Lambert's law predicts that the power absorbed per unit volume, $P^L(z)$ at a given depth z is:

$$P^L(z) = 2I_t\beta e^{-2\beta z} \qquad (1)$$

where I_t is the transmitted power intensity and β is the attenuation factor:

$$\beta = \frac{\pi\sqrt{2\kappa'}\left(\sqrt{\sqrt{1 + \left(\frac{\kappa''}{\kappa'}\right)^2} - 1}\right)}{\lambda_0} \qquad (2)$$

λ_0 is the wavelength in free space, or 12.24 cm at 2450 MHz. The dielectric properties are the real portion, κ', and imaginary portion, κ'', of the relative complex permittivity, κ^*. Lambert's law is strictly valid for semi-infinite samples and requires an estimate of the transmitted power flux.

Power from Maxwell's equations for a slab

Maxwell's equations can be solved for the absorbed microwave power, $P^M(z)$. For a slab of thickness L exposed to radi-

Table 1—Physical and thermal properties of agar gel

Property	Value for 2% agar[a] gel
Specific heat, C_p, J/kg-K	4200
Thermal conductivity, k, W/m-K	.60
Density, ρ, kg/m^3	1070
Dielectric Constant, κ'	73.6
Dielectric Loss, κ''	11.5
Penetration depth, D_p, cm	2.9
Heat transfer coefficient, h, W/m^2°C	4

[a] Difco, Detroit, MI.

ation of intensity E incident from the left face of the sample (Ayappa et al., 1991):

$$P^M(z) = \frac{1}{2} \omega \varepsilon_o \kappa'' E^2 T^2$$

$$\left(\frac{e^{-2\beta z} - 2Re^{-2\beta L} \cos(\delta + 2\alpha(L-z)) + R^2 e^{-2\beta L} e^{-2\beta(L-z)}}{1 - 2R^2 e^{-2\beta L} \cos(2\delta + 2\alpha L) + R^4 e^{-4\beta L}} \right)$$

(3)

$$0 \leq z \leq L$$

where α is the phase factor, β the attenuation factor and κ'' the relative dielectric loss for the slab. ε_o is the free space dielectric constant and $\omega = 2\pi f$, where f is the frequency of radiation. For food samples we assumed that the magnetic permeability was equal to that of free space. T, the transmission coefficient at the sample-air interface, is:

$$T = \sqrt{\frac{4\alpha_o^2}{(\alpha_0 + \alpha)^2 + \beta^2}}$$

(4)

where the subscript 0 denotes air. β is defined in Eq. (2) and α, the phase factor, is:

$$\alpha = \frac{2\pi}{\lambda_s}$$

(5)

where the wavelength within the sample, λ_s, is determined from the dielectric properties of that sample, κ' and κ'':

$$\lambda_s = \frac{\lambda_0}{\sqrt{\frac{\kappa'}{2} \sqrt{\sqrt{1 + \left(\frac{\kappa''}{\kappa'}\right)^2} + 1}}}$$

(6)

The reflection coefficient, R, is:

$$R = \sqrt{\frac{(\alpha_0 - \alpha)^2 + \beta^2}{(\alpha_0 + \alpha)^2 + \beta^2}}$$

(7)

δ, the phase angle for the reflection coefficient, is:

$$\delta = \tan^{-1} \left(\frac{2(\alpha\beta_0 - \alpha_0\beta)^2}{(\alpha_0^2 + \beta_0^2) - (\alpha^2 + \beta^2)} \right)$$

(8)

Power from Combined equation

The Combined equation is obtained from Eq. (3) by taking the limit of the slab thickness L going to infinity. The power, $P^C(z)$, is:

$$P^C(z) = \frac{1}{2} \omega \varepsilon_o \kappa'' E^2 T^2 e^{-2\beta z}$$

(9)

This is a form of Lambert's law that requires a knowledge of the incident field intensity, E, however unlike Lambert's law it incorporates reflections at the incident face of the sample. Equation (9) is referred to as the Combined equation throughout this report.

The incident electric field E in Eq. (3) and (9) is related to the incident power flux I_t of Eq. (1) by:

$$I_t = \frac{1}{2} c \varepsilon_o E^2$$

(10)

where c is the velocity of light.

MATERIALS & METHODS

AGAR GELS WERE PREPARED by dissolving 2% agar (Difco, Detroit MI) in hot distilled water and allowing the gels to cool to 4°C in slabs. The slabs were cut to 7.2 by 3.4 cm to match the entry port on the microwave oven, which was a continuation of the waveguide from the magnetron. The slabs were sliced to a thickness of 0.9 cm or 3.0 cm with a custom cheese slicer.

Gels were heated in a custom microwave oven designed by Hung (1980). The microwave radiation from the magnetron traveled along a waveguide, passed through the entry port and immediately impinged upon the sample. This was done to make the power as unidirectional on the sample as possible. Radiation that passed through the sample without being absorbed was absorbed by a water load placed in the oven cavity. The water load minimized reflections within the oven cavity, which might cause radiation to reflect upon the sample from the back. Oven cavity reflections would make the radiation less unidirectional.

The power level measured by a watt meter in the waveguide, was set to 40 W transmitted and the samples were heated for 7 min. Temperatures were measured along the center axis of the sample, at various depths parallel to the microwave radiation, using phosphor-tipped fiber optic probes (Model 750, Luxtron Corporation, Santa Clara, CA). For the 0.9 cm samples, temperatures were measured every 1 mm, while for the 3.0 cm samples they were measured every 3 mm. The heating rates at one or two locations were measured on each run, with multiple runs averaged to determine the entire temperature-time profile. At least three measurements were taken at each location. The averages and standard deviations were then plotted vs location within the sample.

The dielectric properties were measured using a dielectric probe (model 85070A Hewlett-Packard Co., Santa Rosa, CA.) The specific heat and thermal conductivity were measured by differential scanning calorimetry and a thermal line probe method, respectively. The heat transfer coefficient was calculated by measuring the internal temperature of a block of aluminum, of the same dimensions as the thinnest sample, as it warmed from 4°C to room temperature in still air and plotting the log of the unaccomplished temperature change vs time. The density was determined by weighing the sample and measuring its volume by displacement.

Power and temperature profiles were predicted using a computer model based on Maxwell's equation, Lambert's law or a Combined equation to determine the microwave power term. The Combined equation was the same as Lambert's law with an additional term added from Maxwell's equations to calculate the transmitted power based on incident power. The predictions assumed uniform plane waves incident unidirectionally on a homogeneous infinite slab with no moisture migration (Ayappa et al., 1991).

The assumption of unidirectional radiation on an infinite slab presented some problems. An infinite slab centered within the oven cavity would not receive unidirectional radiation since reflections from the walls would cause radiation to be incident from all directions. This would be a large deviation from the model predictions. An infinite slab placed next to the entry port would receive almost unidirectional radiation, but a large portion of the sample would receive no radiation at all since the sample would be larger than the entry port. This caused large errors due to thermal conductivity to the cold portions of the sample. Thus, a compromise was to use a slab equal in size to the entry port, placed directly next to the entry port. This insured much more uniform radiation, incident across the entire sample surface. Edge focusing effects were minimized because the radiation was only incident on the front face of the sample and not from the sides along the edge.

Lambert's law requires that the power transmitted into the sample be measured experimentally. This was done by measuring the heating rate of a 1 L beaker of water and calculating the power required to heat at that rate. Assuming an isolated system, the absorbed power, P, was P = mC_p ($\Delta T/\Delta t$), where m was 1000g, C_p was 4.19 J/g°C, and $\Delta T/\Delta t$ was the measured heating rate. The power based on this measurement was 4550W. The incident power flux used for the theoretical modeling using Maxwell's and the Combined equations was the 40W of measured incident power, divided by the area of the entry port, or 16400 W/m^2. The physical properties of the agar gel are listed in Table 1.

To determine if there was a statistical difference between predictions and measured temperatures, the χ^2 values were determined (Fienberg, 1981):
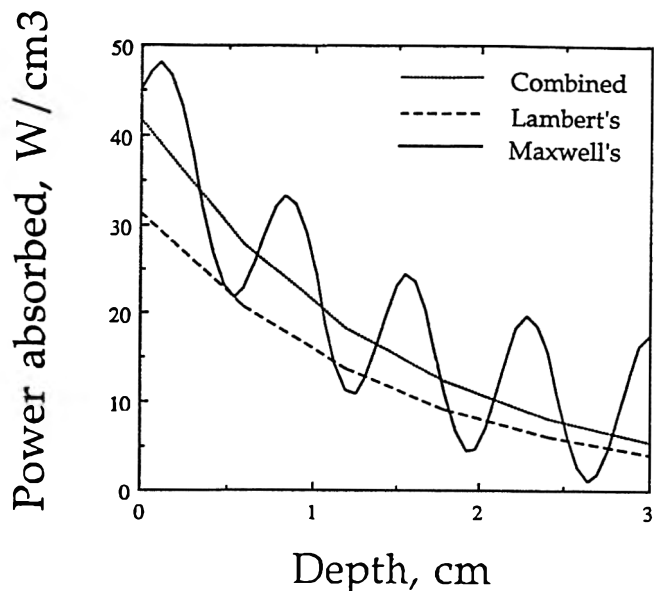
Fig. 1—Theoretical power absorption profiles for a slab of 3.0 cm thickness. The power is incident from the left.
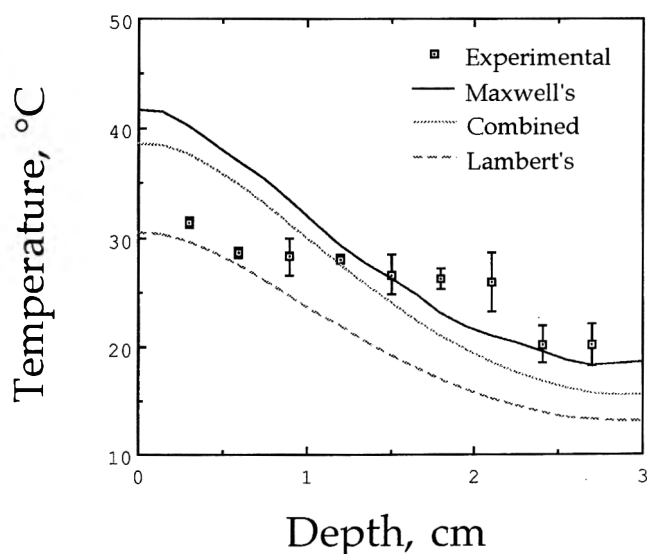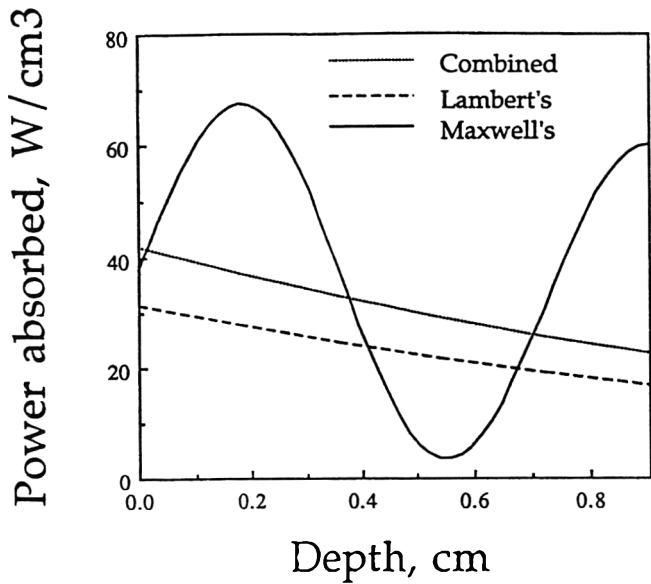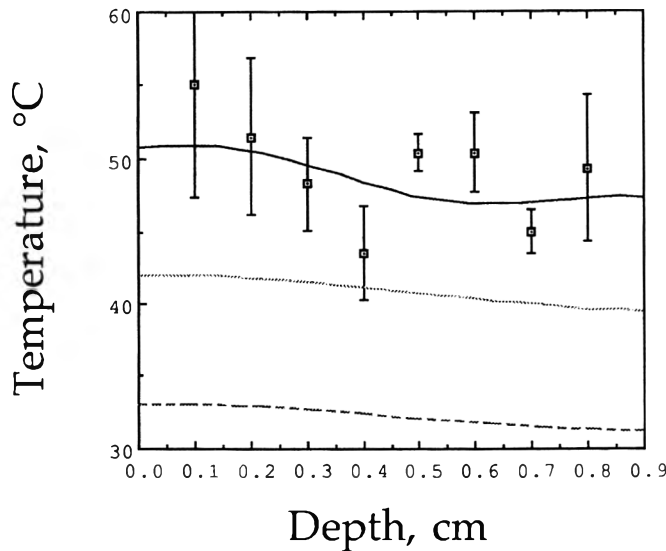

Fig. 2—Theoretical and experimental temperature profiles for a slab of 3.0 cm thickness after 7 min heating. The power is incident from the left.

$$\chi^2 = \sum \frac{(f_o - f_e)^2}{f_e} \qquad (11)$$

where f_o was the observed, or experimental value, and f_e, was the expected, or predicted value. The f values are the difference in temperature between the start and end of heating, °C, at a given location for a given run. The χ^2 value was used to accept or reject the hypothesis that measured and predicted temperatures were the same, at $p \leq 0.05$.

RESULTS & DISCUSSION

Agar gel, 3.0 cm sample

The theoretical power absorption profile, during microwave heating, of a 3.0 cm thick slab of agar was constant throughout the tests because the dielectric properties were assumed to be temperature independent. A cross-section of the sample, (Fig. 1) shows the theoretical power absorbed as related to depth into the sample. Lambert's law predicted an exponential decay of energy with depth into the sample. The Combined equation had a similar shape because it also neglected internal reflections.
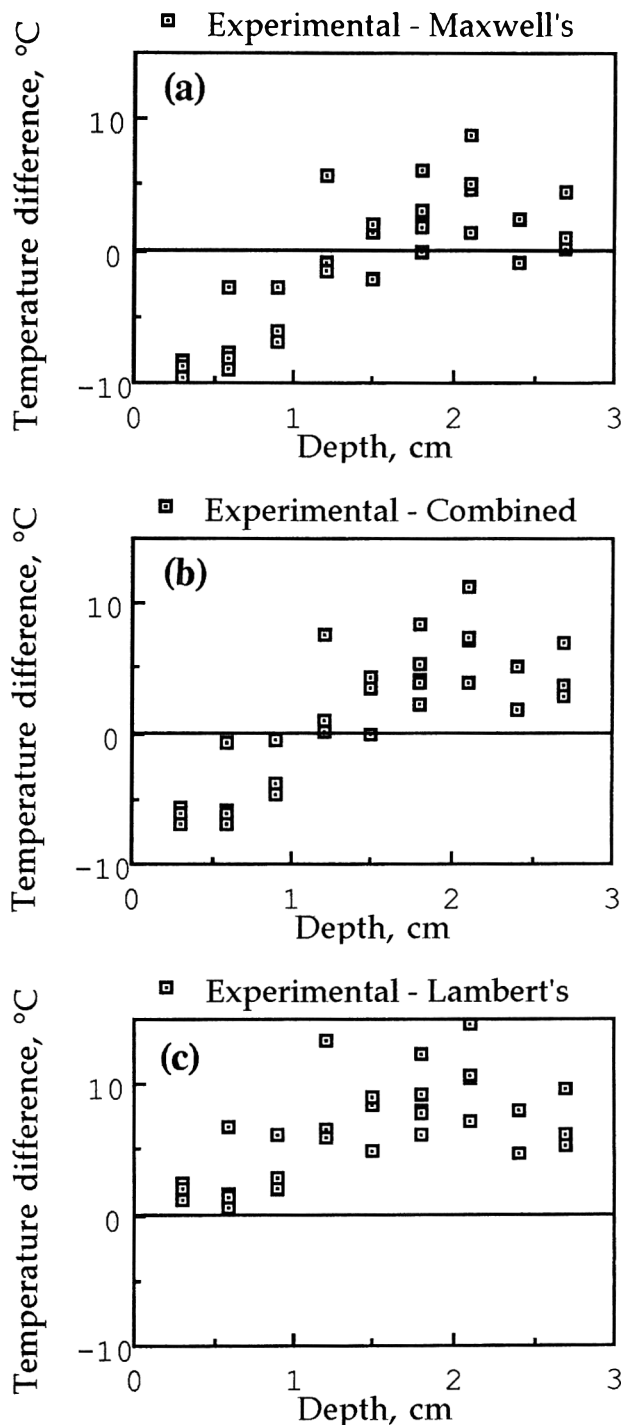
Fig. 3—Temperature differences between measured values in the 3.0 cm samples after 7 min, and those predicted by (a) Maxwell's equations; (b) the Combined equation; and (c) Lambert's law.

However, the curve was shifted upwards because this equation calculates the value for the transmitted power instead of using an experimental value, as for Lambert's law. Maxwell's equations predicted a weak standing wave pattern caused by the reflection of energy from the back surface of the sample. This standing wave approximated the exponential decay predicted by the Combined equation.

From the theoretical power profile, theoretical temperature profiles were calculated for each of the three equations for the period of heating. These temperatures were compared to experimentally measured temperatures. The predicted and measured temperatures were predicted (Fig. 2) at the end of the run, after 7 min heating.

Fig. 4—Theoretical power absorption profiles for a slab of 0.9 cm thickness. The power is incident from the left.

Fig. 5—Theoretical and experimental temperature profiles for a slab of 0.9 cm thickness after 7 min of heating. The power is incident from the left. ▫ Experimental; —— Maxwell; ······ Combined; - - - Lambert's.
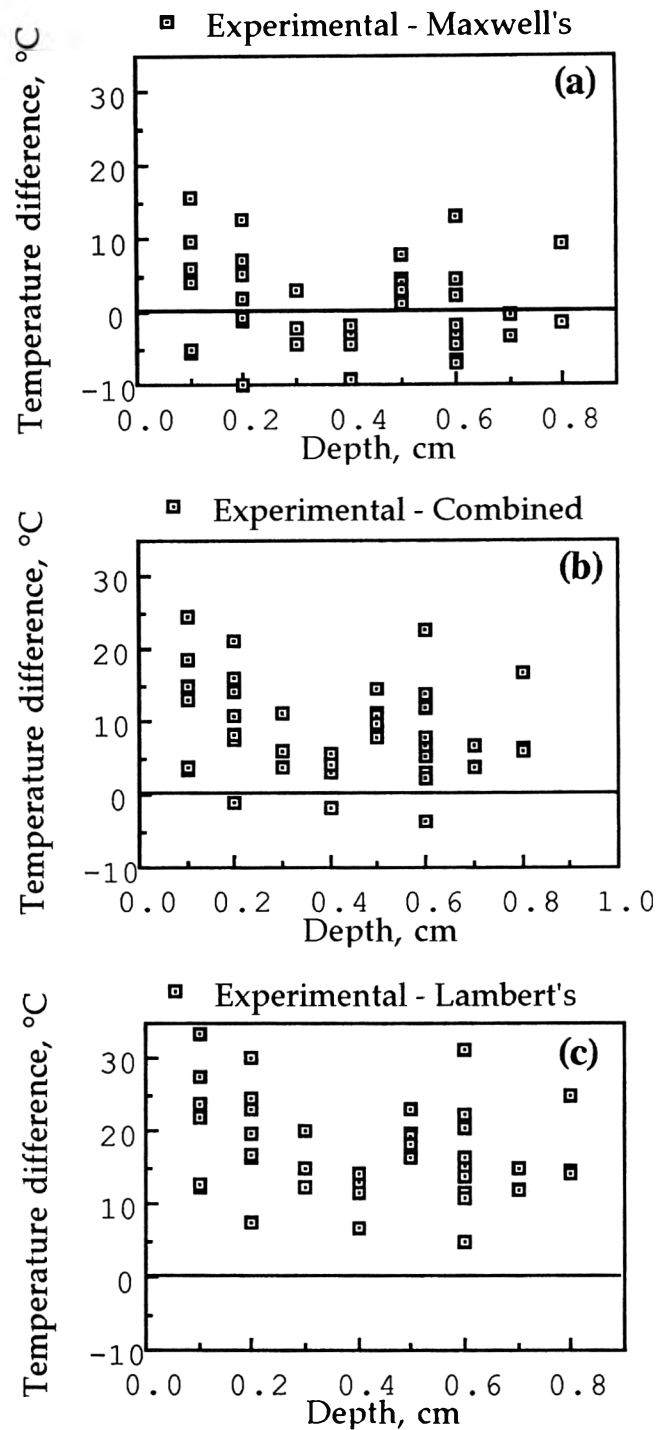
Fig. 6—Temperature differences between the measured temperature values in the 0.9 cm thick slabs after 7 min heating, and those predicted by (a) Maxwell's equations; (b) the Combined equation; and (c) Lambert's law.

The predictions based on Maxwell's equations were close to the measured temperatures for the interior and back surface of the slab. Overall, the Combined equation predicted lower temperatures and the temperatures predicted by Lambert's law were even lower. The χ^2 was used to determine if the difference between experimental and each predicted temperature rise during heating was significant. After 7 min. of heating, $\chi^2_{Maxwell}$ was 30.4, $\chi^2_{Lambert}$ was 131 and $\chi^2_{Combined}$ was 45.9. For a p ≤ 0.05 with 29 degrees of freedom, χ^2 is 42.3. Thus, the Maxwell prediction could not be rejected as different from measured temperatures. The predictions based on the other two equations were statistically different from the measured temperatures. Therefore, Maxwell's equations were the only ones to predict the experimentally verified temperature rise.

The differences between measured temperatures and those theoretically predicted for that time and location were compared (Fig. 3.) A plot of the difference in temperature between experimental tests and predictions based on Maxwell's equations (Fig.

3a), was compared with predictions based on the Combined equation (Fig. 3b) and Lambert's law (Fig. 3c). This figure demonstrates how well the predictions based on equations matched experimental measurements. After 7 min heating, none of the equations predicted the measured temperatures exactly; however, Maxwell's equations predicted 100% of the measured temperatures to ± 10°C, while Lambert's law predicted 83% and the Combined equation predicted 97% to ± 10°C. Additionally, the Lambert law prediction was lower than all experimentally measured temperatures, demonstrating that Lambert's law underestimated the heating rate of thin samples (Fig. 3).

A limitation of the model was that it did not incorporate moisture migration during heating. Since the samples lost weight
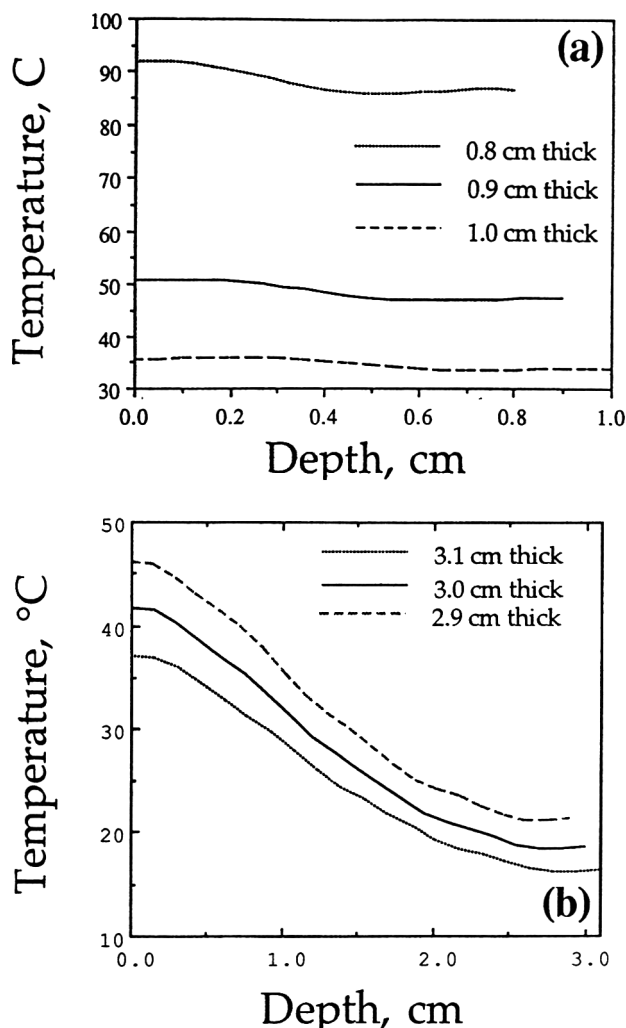
Fig. 7—Predicted temperatures after 7 min of heating for agar slabs varying by up to 1 mm in thickness: (a) for 0.9 cm and (b) for 3.0 cm.

during heating, and appeared dry on the side facing the microwave radiation, loss of water most likely occurred. This loss of water would decrease the dielectric properties and result in a lower heating rate than predicted. If moisture migrated from the front to the back of the sample, the heating rate would decrease at the front of the sample and increase towards the back. Alternately, if moisture migrated from the front of the sample to the air, the heating rate would decrease at the front of the sample. Since the power absorption at the front would be lower, a stronger field would reach the back of the sample, increasing its heating rate. Either situation could explain the observed experimental heating profile.

Agar gel, 0.9 cm sample

The theoretical power absorption profile, during microwave heating, for a 0.9 cm thick slab of agar was evaluated (Fig. 4). The predicted power absorption profiles for the Combined equation and Lambert's law were identical for the 0.9 and 3.0 cm samples (Fig. 1 and 4), but the Maxwell prediction was very different for the two samples. In the 0.9 cm agar slab, much of the radiation reached the back surface of the sample unabsorbed, and so was reflected back into the sample, giving rise to a strong standing wave in the power absorption predicted using Maxwell's equations. At the front and back of the sample were predicted peaks of absorption while near the center the power absorption was lower than predictions by Lambert's law or the Combined equation, due to destructive interference. However,

the average power absorption for the sample was much higher for the Maxwell prediction than for the other two because of the power absorbed from the reflected wave.

The power profiles were used to predict temperature profiles throughout the experiment and these temperatures were compared to experimental temperatures. After 7 min of heating, Maxwell's equations predicted much higher temperatures than did the other equations (Fig. 5). The internal temperature profile predicted by Maxwell's equations was nearly constant throughout the sample and did not show the standing wave pattern evident in the power profile, due to the large effect of thermal conductivity in thin slabs. Lambert's law and the Combined equation predicted temperatures lower than any experimentally measured temperatures. After 7 min heating, $x^2_{Maxwell}$ was 36.2, $x^2_{Lambert}$ was 534 and $x^2_{Combined}$ was 137. The χ^2 value (p ≤ 0.05), 43 degrees of freedom, was 58.9. Thus, statistically the Maxwell prediction could not be rejected as different from the measured temperatures, while the other predictions were different.

The difference between each measured temperature and the temperature theoretically predicted for that time and location, was plotted for the 0.9 cm sample (Fig. 6). Lambert's law and the Combined equation predict temperatures much lower than measured experimentally. This underestimation of the temperature rise during heating could be explained by these equations predicting a power absorption profile that was lower than the experimental profiles. This underprediction of the power absorption profile would be caused by neglecting to include the reflection of energy that occurred at the far interface. The Maxwell prediction fell between the highest and lowest measured temperature increases. This indicated that the temperature rise, and thus the power absorption, was predicted in the correct range. After 7 min of heating, Maxwell's equations predicted 91% of the measured temperatures to ± 10°C. Lambert's law predicted only 7% to ± 10°C while the Combined equation predicted 64% to ± 10°C.

The three equations were compared to determine how important reflections were to accurately predict heating in the microwave oven. Lambert's law did not incorporate any reflections and so, as expected, gave the least accurate prediction of experimental temperature rise. The Combined equation incorporated the reflection at the front face, eliminating the need to experimentally determine transmitted power. This equation improved temperature predictions over those by Lambert's law. Maxwell's equations incorporated the reflections at the front and back faces of the sample, providing the most accurate prediction of internal electric field. Maxwell's equations gave the most accurate prediction of heating in the microwave oven.

If internal reflections have a large effect, as predicted by the Maxwell equations, errors in slicing the sample to the correct thickness would create a wide variability in the data. A slightly thicker or thinner sample would have a different internal temperature profile which would greatly increase or decrease the sample heating rate. The predicted temperature profiles within the sample were compared using Maxwell's equations (Fig. 7), after 7 min of heating. In the 0.9 cm sample, decreasing the thickness by 1 mm greatly increased the final predicted temperature profile while increasing thickness decreased the heating rate, but not as much (Fig. 7a). In the 3.0 cm sample, changing the sample thickness by 1 mm had a much smaller effect on predicted temperature (Fig. 7b). Decreasing the thickness increased its heating while increasing the thickness decreased the heating, but the change in predicted temperature profile was not as sharp as for the 0.9 cm sample.

Changing the thickness of the 0.9 cm slab by 1 mm, (thicker or thinner), changed the predicted end temperature by up to 38 °C, or do bled the heating rate. For the 3.0 cm slab, the maximum variation for a 1 mm change in thickness was 4°C. This indicated that for thin slabs, a small change in thickness could make a large difference in final temperature. For thicker samples, a change in thickness had a smaller effect on the internal temperature profile. Lambert's law and the Combined equation

did not predict a dependence on sample thickness because they did not account for the energy reflected from the far interface.

CONCLUSIONS

MAXWELL'S EQUATIONS could be used to accurately predict the final measured temperature profiles in thin slabs. Predictions based on equations that did not incorporate internal reflections, such as Lambert's law or the Combined equation, were in error. Thus it is important, especially in thin slabs, to include internal reflections in theoretical models. Such internal reflections cause slabs of similar thicknesses to heat at very different rates, due to different internal standing wave patterns. The use of Maxwell's equations showed an advantage in predicting temperature profiles, but further work is necessary to incorporate mass transport of fluid into the predictive models. This should make predictions more accurate. These temperature predictions can be useful in formulating samples that would result in more, or less, uniform heating, as required.

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Plasmin Inactivation with Pulsed Electric Fields

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ABSTRACT

Plasmin (fibrinolysin E.C.3.4.21.7), an indigenous enzyme in bovine milk, added to simulated milk ultrafiltrate (SMUF) at 100 µg/mL (pH 6.11 and ionic strength 0.056 M) was treated at 10°C and 15°C with pulsed electric fields (HVPEF) of 15, 30 and 45 kV/cm and number of pulses 10, 20, 30, 40 and 50. The plasmin activity measured using a commercial assay, was reduced 90% after 50 pulses at both 30 and 45 kV/cm and at a processing temperature of 15°C. Similar inactivation was obtained when plasmin (100 µg/mL) in SMUF was heated at 40°C for 15 min. Inactivation of the enzyme depended on the number of pulses applied during treatment, intensity of the applied field, and processing temperature.

Key Words: plasmin, milk, inactivation, pulsed electric fields

INTRODUCTION

PLASMIN (fibrinolysin E.C.3.4.21.7) or milk alkaline protease is an indigenous enzyme in bovine milk (Kaminogawa et al., 1972; Eigel, 1977; Grufferty and Fox, 1988). Plasmin and plasminogen are components of the proteolytic enzyme system in bovine blood. They pass from the blood to the milk via the mammary glands. Activation of plasminogen results in formation of plasmin. Plasminogen activators and plasmin inhibitors also appear in the milk. The activators of plasminogen are associated with the casein micelles in milk while the plasmin inhibitors are found only in the serum phase (Grufferty and Fox, 1988). Plasmin activity results in production of γ-caseins and proteose-peptones from β-caseins (Bastian et al., 1991; Eigel, 1977; Kohlmann et al., 1991). The concentration of plasmin in milk is related to several factors including the stage of lactation, breed of cows, age of cows, mastitis and storage temperature (Richardson, 1983b; Grufferty and Fox, 1988; Politis et al., 1989a, b).

The proteolytic activity of plasmin promotes several changes in milk. These include a decrease of viscosity of casein dispersions prepared from milk (Grufferty and Fox, 1988) and an increase in the amount of soluble protein due to the formation of peptides (Grufferty and Fox, 1988). An increase in rennet coagulation time also occurs with an increase in β-casein hydrolysis during cheese ripening (Humbert and Alais, 1979; Richardson and Pearce, 1981) and gelation of UHT milk (Grufferty and Fox, 1988; Kohlmann et al., 1991). Pasteurization decreases initial plasmin activity in milk (Korycka-Dahl et al., 1983; Richardson, 1983a), but the proteolytic activity increases during storage of processed milk (Humbert and Alais, 1979; Korycka-Dahl et al., 1983; Richardson, 1983a). The increase in proteolytic activity can be explained by the destruction of inhibitors of plasminogen activators during heating (Richardson, 1983a). The use of β-lactoglobulin as an inhibitor of plasmin has been reported by Bastian et al. (1993).

Factors which affect activity of the enzyme are temperature, reaction time, enzyme concentration, nature of substrate, and thermostability of the enzyme and inhibitors (Visser, 1981). Inactivation of plasmin in milk is a function of pH during heating,

decreasing plasmin stability with an increase in pH (Richardson, 1983a; Grufferty and Fox, 1988). Kaminogawa et al. (1972) reported a rapid decrease of plasmin activity after heating a buffer solution (pH 7.0) containing the enzyme at 40 to 80°C for 10 min. In addition, the stability of plasmin is lower in a non-micelle system than in a micellar casein dispersion (Grufferty and Fox, 1988).

In the 1920s and 1930s, the use of electric treatments to pasteurize milk was widely studied (Beattie and Lewis, 1925; Fetterman, 1928; Moses, 1938). The earlier objective was to heat the milk by passing current through the product (ohmic heating). Sale and Hamilton (1967) demonstrated the nonthermal lethal effect of electric fields on bacteria such as *Escherichia coli, Staphylococcus aureus, Micrococcus lysodeikticus, Sarcina lutea, Bacillus subtilis, Bacillus cereus, Bacillus megaterium*, and *Clostridium welchii*, and yeasts such as *Saccharomyces cerevisiae* and *Candida utilis*. Similar results were reported by Sato et al. (1988), Mizuno and Hori (1988), Zhang et al. (1995) and Pothakamury et al. (1995).

The main effect of an electric field on microorganisms is an increase in membrane permeability because of compression and poration. Cell breakdown or inactivation is achieved because of osmotic imbalance across the cell membrane induced by poration. Reduction of up to 9 log cycles (9D) for *Escherichia coli* (*E. coli*) was obtained with pulsed electric fields of 50 kV/cm (Zhang et al., 1995).

Gilliland and Speck (1967) reported the inactivation of trypsin and protease from *B. subtilis* using an electric field of 31.5 kV/cm. Castro (1994) reported the inactivation of alkaline phosphatase in simulated milk ultrafiltrate (SMUF) by applying an electric field of 20 kV/cm. The inactivation mechanism for the enzymes proposed by Gilliland and Speck (1967) was an oxidative reaction of key components induced by electric fields as a function of treatment time.

Our objective was to examine the feasibility of using pulsed electric fields (PEF) in the inactivation of plasmin in simulated milk ultrafiltrate based upon limited information available on the effects of PEF on enzymatic activity. The variables included number of pulses, electric field strength and processing temperature.

MATERIALS & METHODS

Plasmin solution and simulated milk ultrafiltrate

Plasmin E.C.3.4.21.7 from bovine plasma (Sigma Chemical, St. Louis, MO, 80 mg freeze-dried sample) was reconstituted with 10 mL of 1 mM HCl and frozen in 1-mL vials at −35°C until used. Simulated milk ultrafiltrate (SMUF) provided a casein micelle-free medium with electrical and chemical properties similar to milk. SMUF consists of lactose (50 g), potassium phosphate monobasic (1.58 g), tri-potassium citrate (0.98 g), tri-sodium citrate (1.79 g), calcium chloride (1.32 g), magnesium citrate (0.38 g), potassium carbonate (0.30 g), and potassium chloride (1.08 g) in 1L deionized water. SMUF was diluted with deionized water to modify electrical properties in the proportion 1:2 (v/v). The solution was filtered through a 0.22-µm sterile filter and stored in sterile bottles. The pH of SMUF was 6.11 with an ionic strength of 0.056 M. An experimental SMUF/plasmin solution was prepared by mixing a portion of the reconstituted plasmin solution and diluted SMUF to a final concentration of 100 µg/mL plasmin. The solution was stored at 4°C while inactivation experiments were conducted.

Pulsed electric fields (PEF)

A continuous flow chamber (Fig. 1) consisting of two parallel stainless steel electrodes and a polysulfone spacer was used to apply the PEF
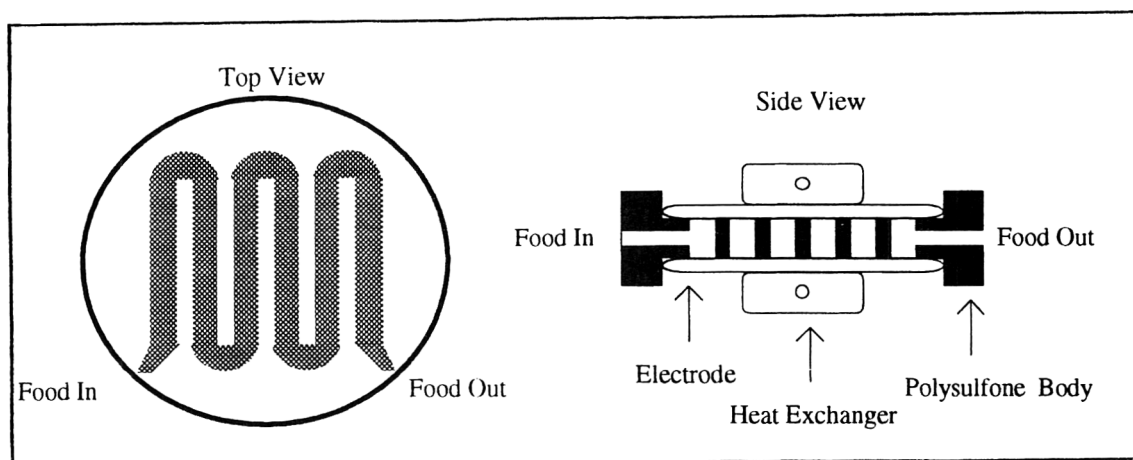
Fig. 1—High voltage continuous treatment chamber.

Actual Plate

Well No.	Description
1, 2	50 pulses
3, 4	Control
5, 6	10 pulses
7, 8	20 pulses
9, 10	30 pulses
11, 12	40 pulses

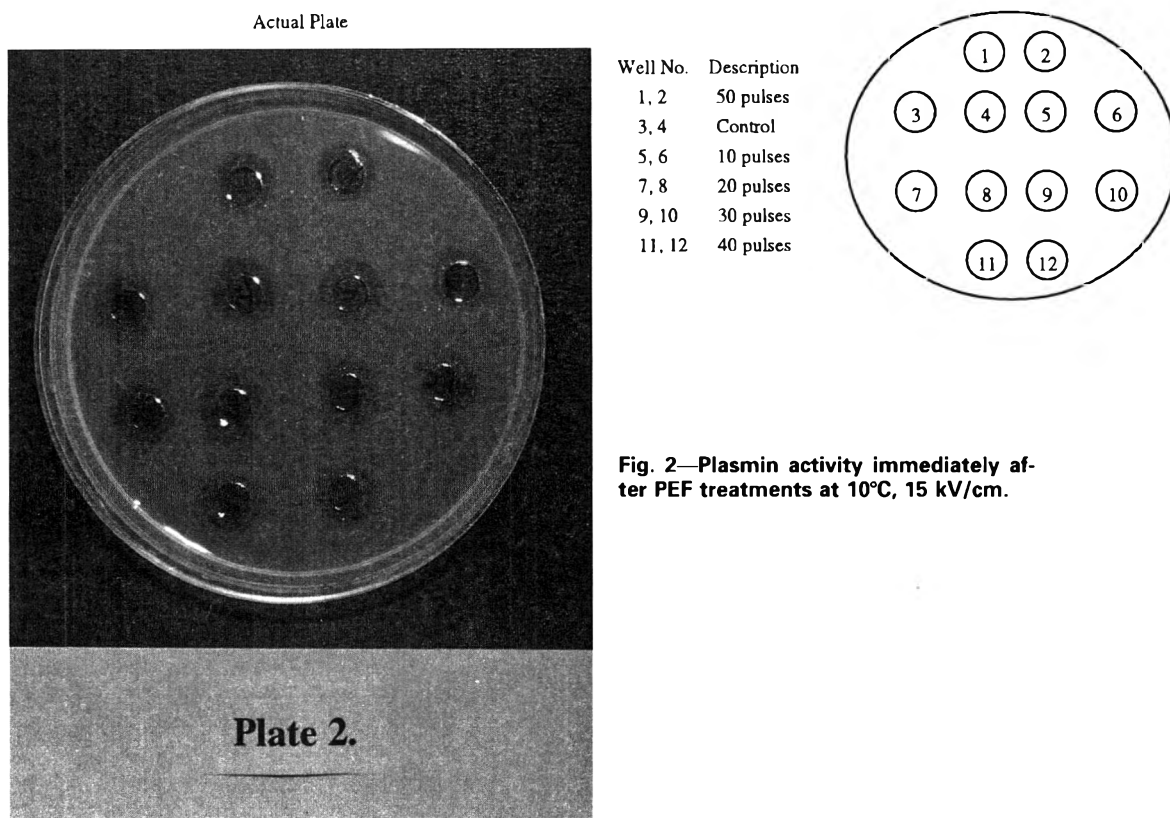Plate 2.

Fig. 2—Plasmin activity immediately after PEF treatments at 10°C, 15 kV/cm.

treatments. The temperature was controlled by passing cooling water through built-in heat exchangers on the electrodes. SMUF (15 mL) was placed in a closed-loop system consisting of a reservoir, peristaltic pump, and a chamber.

Experimental conditions were: constant flow rate: 45 mL/min; processing temperature: 10 or 15°C measured using a thermocouple attached to the surface of the heat exchanger; electric fields: 15, 30 or 45 kV/cm measured with an oscilloscope (Hewlett Packard 54520A, Colorado Springs, CO); number of pulses: 10, 20, 30, 40 or 50; pulse rate: 1 pulse/10 sec (0.1 Hz); and, pulse duration: 2 μsec.

Thermal inactivation of plasmin

A sample of SMUF/plasmin solution (5 mL) was placed in a test tube and heated at a preselected temperature of 40, 60 or 80°C. The temperature was measured with a glass thermometer immersed in the solution. Plasmin activity was determined after 5, 10, 15, 20, 25, 30 and 50 min during the heating period. An aliquot of 100 μL was removed at the specified intervals, cooled in ice, and immediately tested for activity.

Relative activity of plasmin

The proteolytic activity of plasmin was determined using Bio-Rad Protease Activity Gel Tablets™ (BIO-RAD, Life Science Group, Richmond, CA). Two gel tablets were dissolved in 15 mL water. The solution was heated in boiling water for three min until all agar was melted; then it was poured into a standard polystyrene 100 × 15 mm petri dish. The gel contained bovine casein in Tris-buffered saline at pH 7.4. Twelve wells of 6.35 mm diameter were punched in the gel using a glass tube.

Plasmin solution (25 μL) was placed in the wells and incubated at 25°C for 24 hr. Acetic acid (3% v/v) was used to precipitate the casein in the gel and to enhance the clear zone around the wells produced by plasmin activity. The change in plasmin activity was determined as change in capacity of the enzyme to hydrolyze casein in the gel, which was related to the width of clear zone in the gel. The reduction in proteolytic activity was estimated as the % reduction in clear zone (%RCZ):

$$\% \, RCZ = (D_{in} - D_{tr})/(D_{in} - D_{well}) \tag{1}$$

where D_{in} is the clear zone diameter of 100 μg/mL plasmin control, D_{tr} is the diameter of the clear zone for treated plasmin after 24 hr incu-
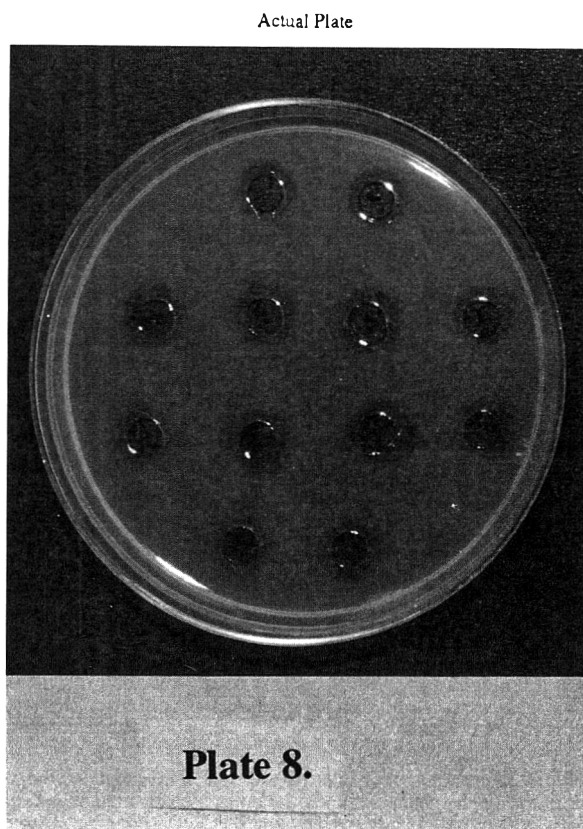
Actual Plate

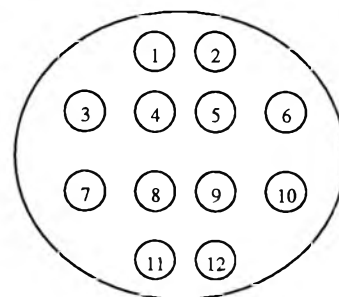| Well No.	Description
1, 2	50 pulses
3, 4	Control
5, 6	10 pulses
7, 8	20 pulses
9, 10	30 pulses
11, 12	40 pulses

Fig. 3—Plasmin activity immediately after PEF treatments at 15°C, 15 kV/cm.
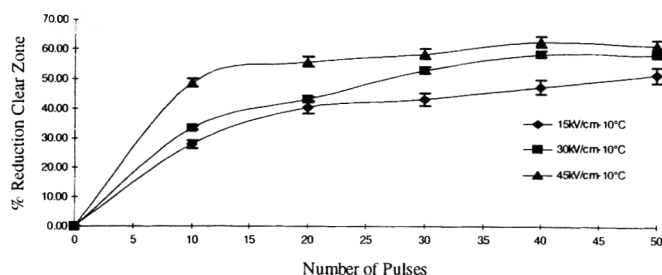
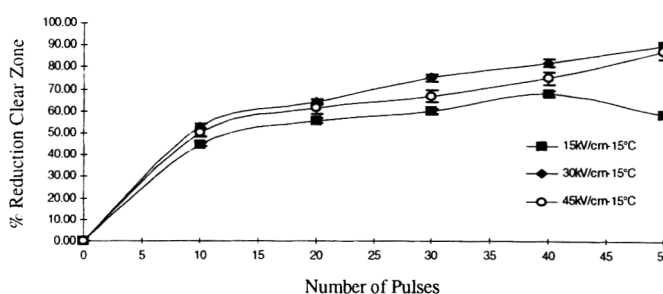Plate 8.

Fig. 4—PEF inactivation of plasmin at 10°C.

Fig. 5—PEF inactivation of plasmin at 15°C.

bation, and D_{well} is the well diameter. A completely random design was used for collection of experimental data. Each inactivation test was replicated, and each time two wells were used to test activity of the enzyme. Data were analyzed using the general linear model of SAS© (SAS Institute, Inc., 1990).

RESULTS & DISCUSSION

PEF inactivation of plasmin

Examples of the effects of PEF on plasmin activity were compared (Fig. 2 and 3) where the activity of the enzyme was characterized by color changes in the casein gel. The clear zones around the wells represented the solubilization of casein by plasmin after 24 hr incubation at 25°C. Plasmin activity, expressed as a function of the clear zone size decreased 90% when treated at both 30 and 45 kV/cm, 15°C and 50 pulses. The reduction in clear zone size, which followed first-order reaction kinetics, increased with an increase in number of pulses, temperature, and electric field (Fig. 4 and 5). Analysis of variance demonstrated that number of pulses, electric field and temperature were significant in the inactivation of plasmin (p ≤ 0.01). These results suggested a synergistic effect between selected factors in the inactivation rate of plasmin.

PEF-treated plasmin solutions showed no significant changes in activity after 24 hr storage at 4°C (Table 1) which suggested a permanent inactivation of the enzyme when exposed to pulsed

Table 1—Activity of plasmin after PEF treatments

Treatment kV/cm-°C	Clear zone diameter (mm)[a]	
	A	B
Control	13	13
15-10	10	10
30-10	9	9
45-10	9	8
15-15	10	10
30-15	7	8
45-15	7	7

[a] A—Assayed immediately after PEF treatment, 50 pulses. B—Stored 24 hr at 4°C after PEF treatment prior to assay.

electric fields. The inactivation mechanism of plasmin under PEF may be explained in terms of charge and configuration changes due to the electrostatic nature of plasmin as a protein (Sharp and Honig, 1990; Robertson and Astumian, 1990).

Thermal inactivation of plasmin

The thermal inactivation of plasmin in SMUF (pH 6.11 and I = 0.056 M) was obtained within 5 min at 60 and 80°C while 15 min were required at 40°C (Fig. 6). Results were very specific for the experimental conditions considered in this work because SMUF is an ionic, casein micelle-free medium. The absence of
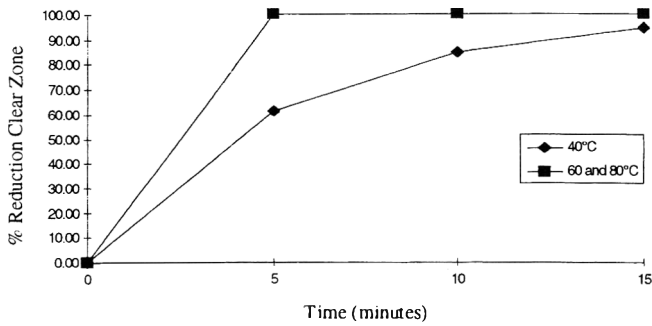
Fig. 6—Thermal inactivation of plasmin at 40, 60 and 80°C.

the casein micelle would decrease heat stability of the plasmin (Grufferty and Fox, 1988). Note that the experimental solution (SMUF/plasmin) only contained plasmin and not plasminogen. The latter would have promoted proteolytic activity during heat treatments due to inactivation of plasmin inhibitors in systems such as milk (Humbert and Alais, 1979; Grufferty and Fox, 1988). Thermal inactivation was more likely related to conformational changes and chemical reactions within the enzyme when heated (Ahern and Klibanov, 1985)

The temperature-inactivation relationship during PEF treatment of plasmin was different from relationships observed during thermal inactivation. Processing temperatures ($\leq 15°C$) below room temperature (vs 40 to 80°C during thermal treatment) were suitable to inactivate plasmin using PEF with a total treatment time of 100 μsec. Thus, the inactivation of plasmin using PEF, as we performed it, could be considered a nonthermal process.

CONCLUSION

PLASMIN ACTIVITY was reduced when exposed to high-intensity pulsed electric fields. Reduction of proteolytic activity of plasmin, in an ionic medium such as SMUF, is a function of number of pulses, electric field strength and processing temperature. The most important aspect of such treatment was that the inactivation occurred at 15°C which could be considered a nonthermal process compared to inactivation of plasmin at 40°C for 15 min using heat treatments. Pulsed electric fields have the potential for inactivation of proteolytic enzymes in foods.

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Ms received 4/23/94; revised 3/25/95; accepted 4/16/95.

Degradable Wheat Gluten Films:
Preparation, Properties and Applications

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ABSTRACT

Degradable films from spray-dried (SD) and flash-dried (FD) wheat gluten prepared at various pHs, salt levels, temperatures, and shearing times were compared. Differences were observed in properties of the films related to processing conditions. Films prepared from SD wheat gluten were thicker, and had higher tensile strengths and Young's moduli. SD wheat gluten solutions applied as coating material resulted in Grade A-quality shell eggs maintaining quality for 30 days at room temperature. Additionally, SD wheat gluten coating increased the water stability of starch-based packaging.

Key Words: wheat gluten, films, biodegradable, shearing time

INTRODUCTION

A COST-EFFECTIVE DEGRADABLE FILM or coating that extends food product shelf life and preserves quality may have advantages in the food industry. Films are used in the confectionery, fruits and vegetables, meat, and pharmaceutical industries. One research area involving edible film application is multilayer barriers (Fennema et al., 1993). Such technology may be useful for multicomponent perishable food items such as pizza and pie. Separation of frozen dough from tomato sauce partitioned by a layer of edible film improves product quality by reducing contact and moisture transfer during frozen storage.

Several polymers, including polysaccharides, proteins, lipids or a combination have been used to produce edible films and coatings (Kester and Fennema, 1986; Gennadios and Weller, 1991; Avena-Bustillos and Krochta, 1993; McHugh et al., 1994). However, a major disadvantage of these films is their high water vapor permeability that is undesirable in edible coatings or packaging materials. Wheat proteins contribute both elastic and cohesive properties that are useful characteristics for non-food products such as garbage bags (Wall and Beckwith, 1969; Aydt et al., 1991). Wheat proteins also can be utilized as edible coatings for several food applications. In shell eggs, coatings prepared from degradable polymers and oils impeded microbial invasion and increased shell strength (Meyer and Spencer, 1973; Tryhnew et al., 1973). Degradable films and coatings from wheat gluten may provide new channels for marketing wheat gluten.

Researchers have reported spray-dried (SD) wheat gluten as a source for degradable films (Gennadios and Weller, 1992; Gennadios et al., 1993). However, flash-dried (FD) wheat gluten has not been reported as a material for preparation of degradable films and coatings. The flash-drying process produces larger size particles that may form films with different mechanical and barrier properties. The objectives of our study were to compare mechanical and barrier properties of films using SD and FD wheat gluten and to investigate applications of such wheat gluten films.

MATERIALS & METHODS

Gluten film preparation

SD and FD wheat gluten samples were donated by Midwest Grain Products, Inc. (Atchison, KS). The particle size of the wheat gluten was determined by passing it through a 200 mesh screen. About 98% of SD wheat gluten and 70% of FD wheat gluten passed through the 200 mesh screen. Overall, particle size of SD wheat gluten was smaller than that of FD wheat gluten. Films were prepared from a solution of 18% gluten (18g/100 mL w/v), 85 mL 95% ethanol, 45 mL distilled deionized water (ddH$_2$O), 6.2g glycerol, and either 0.1 or 0.5M magnesium sulfate (MgSO$_4$). The solution was stirred in a beaker on a hot plate for 5 min, and the pH of the solution was adjusted to either 3.3 with glacial acetic acid or 10.0 with 6N ammonium hydroxide. The film-forming solution was sheared for 5 or 10 min using a Brinkmann Homogenizer on setting 4 (model PT 10/35, Switzerland). The sheared film-forming solution was heated while stirred to 40°C or 80°C and was centrifuged (model CL, International Equipment Co., Needham Heights, MA) at speed setting 3 for 5 min. Centrifugation removed insoluble gluten, thus improving film clarity. Protein solubility may influence film formation. Variables studied included pH, salt level, shearing and temperature. Film-forming solution supernatant (50 mL) was immediately cast on a rectangular polyethylene mold (15 cm × 28 cm). The film-forming solution was dried overnight at room temperature, and films were peeled manually on the following day.

Film thickness

Film thickness was measured using a micrometer (Fisher Scientific, Pittsburgh, PA). Film strips were placed within the micrometer, and the gap reduced until first indication of contact was noted. Measurements were taken at five different locations on the film ($\pm$ 10 µm) and the mean value was used to calculate apparent water vapor permeability (WVP$_{app}$) and tensile strength.

Electrophoresis

Film-forming solutions were concentrated on a rotory evaporator (Buchi R-110, Brinkmann Instruments., Westbury, NY). During evaporation, the water bath was kept at room temperature with the flask rotating on a medium/high setting. The film-forming solutions were maintained on the evaporator until emission of volatiles ceased, resulting in a thick and viscous protein solution. From each concentrated sample, a 1-g sample was removed for protein determination using the Kjeldahl method (AOAC, 1988).

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed using a II Dual Slab Cell (Bio-Rad Laboratories, Richmond, CA) and following the procedure of Laemmli (1970). Gels were stained with Coomassie blue R-250 for 30 min and destained using a solution of ddH$_2$O:methanol:acetic acid (5:4:1) for at least 3 hr.

Apparent water vapor permeability (WVP$_{app}$)

Degradable film samples were placed over the open mouth area of metal cups (open mouth area of cup = 33 cm^2) and secured between a rubber gasket and a metal plate to permit water vapor transfer. After initial weights were recorded, the cups were placed in a temperature- and humidity-controlled chamber (model 3940, Forma Scientific, Inc., Marietta, OH). Measurements were taken at 30-min intervals for 10 hr at 23°C and 55% relative humidity (Labuza and Contreras-Medellin, 1981; Park and Chinnan, 1990). WVP$_{app}$ was calculated by the method reported by McHugh et al. (1993).
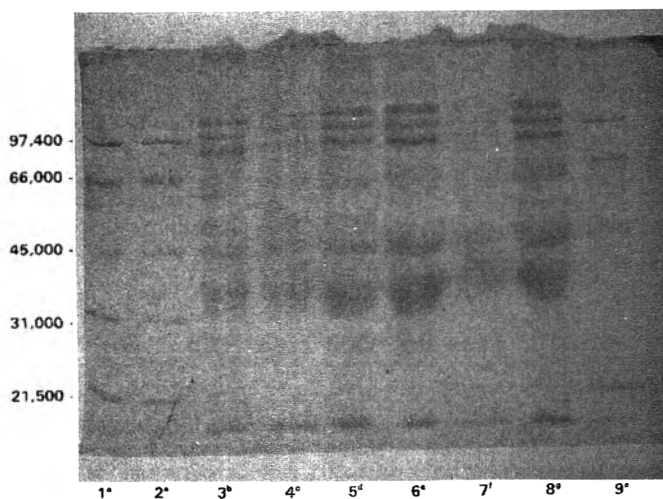
Fig. 1—Electrophoregram of spray-dried (SD) and flash-dried (FD) wheat gluten film-forming solutions resolved on 12% polyacrylamide gel.

[a]1, 2, 9 = molecular weight standards
[b]3 = FD, pH 6.8
[c]4 = FD, pH 3.3
[d]5 = FD, pH 10.0
[e]6 = SD, pH 10.0
[f]7 = SD, pH 3.3
[g]8 = SD, pH 6.8

Tensile strength and Young's modulus

Tensile strength was calculated from peak stress divided by the film cross sectional area. Young's modulus was calculated by the ratio of stress over strain at zero slope. Barbell-shaped strips 110 mm long × 25 mm wide with a 10 mm tapered width at the center were cut from the sample film. An Instron Universal Testing Instrument (model no. 4502, Instron Engineering Corp. Canton, MA) was used to measure the amount of uniaxial force necessary to pull each strip apart. Film strips were placed between grips on the universal testing machine. Initial grip separation was 35 mm, with a crosshead speed of 25 mm/min.

Applications

Egg shell coating. One-day-old eggs from Single Comb White Leghorn hens were used. The treatments were a control (uncoated) and coatings of mineral oil or SD wheat gluten protein. Based on the film's physical and barrier properties SD wheat gluten was used in the application. Oil-coated shell eggs were supplied by a local egg processor. Three eggs for each replication were immersed for 1 min in a 250 mL beaker containing the pH 10 SD wheat gluten coating solution. Haugh unit index, a measure of egg internal quality, was measured on days 1, 3, 5, 7, 10, 14, 21, and 28 at room temperature.

Degradable packing material. Starch-based packing material (Kansas State University, Manhattan, KS) was coated with a SD wheat gluten solution (pH 3.3). After the solution was applied via an aerosol bottle, the "peanuts" were allowed to dry for 48 hr at room temperature prior to analysis. A texture analyzer (model TAXT2, Texture Technologie Corp., Scarsdale, NY) was used to determine the strength of the starch-based coated particles. The probe was lowered at 0.5mm/sec until the "peanut" was punctured, and the force in grams was recorded. The cylindrical-shaped, starch-based, coated "peanuts" deteriorated to a flat and fragmented mass after immersion in water. The time to reach this qualitative condition was considered a measure of the water stability of the peanuts.

Statistical analysis

Each replication included preparation and triplicate evaluation of films at all levels of factorial combinations (pH, salt level, temperature, and shearing time). Three replications were performed; each replication represented a block. Mean comparisons were made when main effects were significant (P < 0.05). A completely randomized design was followed for studies involving applications. Least square means procedure was used to compare means when the effects were significant P < 0.05) (SAS, 1985).

RESULTS & DISCUSSION

Protein content

SD and FD wheat gluten contained 71.66 and 69.25% protein. respectively. SD wheat gluten film-forming solutions at pH 3.3 contained 6.51% protein and at pH 10.0 had 10.52% protein. The FD solutions of wheat gluten contained 6.03% protein at pH 3.3 and 9.68% at pH 10.0. The FD and SD film-forming concentrates prepared for electrophoresis had similar protein contents of ≈60.67% at pH 3.3 and 40.38% at pH 10.0. The lower protein content in the film-forming solutions compared to the protein concentrates was attributed to the insoluble SD or FD wheat gluten that was centrifuged out prior to casting. The majority of the insoluble protein might have been glutenin, which is insoluble in ethanol.

Electrophoresis

FD and SD wheat gluten film-forming solutions had gliadin bands similar to those reported by Lookhart and Albers (1988). Because gliadin is ethanol soluble and glutenin is not, gliadin is assumed to be the major contributor to film formation. In addition, Lookhart and Albers (1988) indicated that higher molecular weight glutenins were attached to gliadin and, therefore, were not removed during initial purification steps. FD wheat gluten film-forming solutions at pH 3.3 exhibited a less intense band at ≈MW 100,000 than those at pH 10.0 indicating a loss of solubility of that fraction at the lower pH. SD wheat gluten film-forming solutions at pH 3.3 did not exhibit a band at ≈MW 66,000, and the 3 bands in the range MW 10.000 to 200,000 were less intense (Fig. 1). The FD and SD wheat gluten film-forming solutions exhibited less intense bands at pH 3.3 than at pH 10.0. This suggested that a decrease in protein solubility occurred under acidic conditions as a result of protein denaturation.

Film thickness

Effects of main factor variables (pH, salt concentration, temperature, shearing time) on the properties of FD and SD wheat gluten films were compared (Table 1). Films prepared from both sources at pH 10.0 were thicker than those from pH 3.3 solutions. The film solutions prepared from SD wheat gluten with 0.5M MgSO$_4$ produced thicker films than those made with 0.1M MgSO$_4$. The higher salt concentration provided additional ions that reacted with the gluten protein and decreased electrostatic attraction between protein molecules. Wall and Beckwith (1969) reported that wheat gluten was insoluble in water at neutrality, but could be dissolved in acid or alkali at low ionic strengths. Increased solubility at higher ionic strength (0.5M or 0.1M) might be the reason for increased thickness of SD wheat gluten films. However, such effect was not observed in FD wheat gluten films, probably due to decreased solubility of FD wheat gluten. Neither temperature nor shearing time influenced film thickness of FD wheat gluten films.

Tensile strength and Young's modulus

Both FD and SD wheat gluten films prepared at pH 3.3 had more tensile strength than those prepared at pH 10.0. This correlated with improvement in dispersion of gluten proteins in the film solutions, especially glutenin. At a lower pH, fewer proteins are dissolved, and molecular structures unfold because of a surplus of positive electrostatic charges (Wall and Huebner, 1981). Higher salt concentration (0.5M) and higher temperature (80°C) resulted in a decrease in tensile strength for both FD and SD wheat gluten films. Salting out of proteins can occur at salt levels of 0.5M. The reduction in protein solubility at 0.5M

Sample	Treatment	Film thickness (μm)	Tensile strength (MPa)	Young's modulus (MPa)	WVP$_{app}$[c] E-9 (g/msPa)
FD[d]	pH				
	10.0	209[b]	0.49[a]	7.22[a]	1.028[a]
	3.3	118[a]	1.82[b]	16.90[b]	5.096[b]
	MgSO$_4$				
	0.1M	162	1.35[b]	10.67	8.281
	0.5M	166	0.96[a]	13.46	7.097
	Temperature				
	40	156	1.55[b]	12.35	6.582
	80	173	0.76[a]	11.77	8.797
	Shearing time				
	5 min	162	0.97[a]	8.15[a]	8.700
	10 min	166	1.34[a]	15.97[b]	6.677
SD[d]	pH				
	10.0	212[b]	0.94[a]	17.57[a]	4.947[a]
	3.3	168[a]	3.41[b]	28.74[b]	8.85[b]
	MgSO$_4$				
	0.1M	178[a]	3.29[b]	25.11	8.45
	0.5M	202[b]	1.06[a]	21.20	5.35
	Temperature				
	40	185	2.67[b]	27.82	7.67
	80	195	1.68[a]	18.49	6.12
	Shearing time				
	5 min	180[a]	2.38[a]	17.55[a]	5.73
	10 min	200[b]	1.97[a]	28.76[b]	8.07

[a,b] Mean values in the same column with different superscripts for each sample and treatment are significantly different (P < 0.05)

[c] WVP = Water Vapor Permeability

[d] FD and SD are flash-dried and spray-dried wheat gluten, respectively.

Table 2—Comparison of specific mechanical and barrier properties of plastic wrap and flash-dried (FD) and spray-dried (SD) wheat gluten films

Parameter	FD film	SD film	Plastic Wrap
Film thickness (μm)	167[b]	189[c]	30[a]
WVP$_{app}$[d] (g/msPa)	7.70 × 10^{-9a}	7.10 × 10^{-9a}	3.09 × 10^{-13b}
Tensile strength (MPa)	1.19[b]	2.12[c]	0.0643[a]
Young's modulus (MPa)	12.32[a]	24.67[b]	11.83[a]

[a-c] Mean values in the same row followed by different superscripts are significantly different (P < 0.05).

[d] WVP = Water vapor permeability.

MgSO$_4$ reduced the amount of protein in the film-forming solution and subsequently decreased film tensile strength. Reduction in tensile strength of wheat gluten protein at 80°C may be a result of plasticizers decreasing intermolecular forces along the protein molecule. High temperature reduces hydrogen and hydrophobic bonding leading to protein unfolding. Greater opportunity for plasticizer distribution throughout the film and subsequent reduction in tensile strength might occur. Conversely, Gennadios and Weller (1992) reported tensile strength measured at 40°C was greater than at 25°C because more water evaporated, decreasing the potential for water to act as plasticizer. At 40°C less protein unfolding occurs; therefore, water acting as a plasticizer would be of greater importance. An increase in shearing time from 5 to 10 min did not affect tensile strength of films. Gennadios et al. (1993) reported that the tensile strength was higher at pH 10.0 than at pH 3.3. Differences in film thickness and glycerol concentration may have been responsible for the contrasting results.

Films prepared from both FD and SD wheat gluten at pH 10.0 were two times more elastic than those prepared at pH 3.3 (Table 1). The smaller Young's modulus, the greater the elastic deformation for a given stress. In an alkaline environment, disulfide bonds are reduced to sulfhydryl groups, thus allowing the film to stretch further. Gennadios and Weller (1992) reported that elasticity was affected in an alkaline pH. This basic structural change in gluten proteins might affect other properties of films. The loss in the electrophoretic band at 66,000 MW at pH 3.3 might have been responsible for the difference in mechanical properties. Gontard et al. (1992) reported that wheat gluten solutions with pH <4.0 produced softer films with lower cohesive structures. Film-forming solutions sheared for 10 min prior to casting exhibited a significant increase in Young's modulus.

This might have been a result of an increase in polymerization. The distribution of plasticizer and its interaction with polymers affect certain mechanical properties of films. Gennadios et al. (1993) reported similar trends using different glycerol levels. Glycerol decreases intermolecular interactions that might result in less brittle films. Temperature and salt level did not affect Young's modulus. The flash-drying process produced larger particles and decreased the packing density. Therefore there was less particle to particle interaction which decreased elastic properties and increased plasticity. McHugh and Krochta (1994) concluded that type and level of plasticizer influenced tensile properties of whey protein films. Temperature did not affect the mechanical properties. Increasing shearing time from 5 to 10 min resulted in a significant increase in Young's modulus.

SD wheat gluten films exhibited a higher tensile strength than did the FD wheat gluten films (Table 2). Higher solubility of SD wheat gluten compared to FD wheat gluten was expected due to relatively lower heat denaturation of SD wheat gluten during the drying process. A better polymer orientation during film formation due to increased solubility might result in conditions conducive for protein-protein interactions. This may be the reason for higher tensile strength for SD films. Brandenburg et al. (1993) reported that alkali-treated soy protein isolate gave higher elongation and improved film appearance. Modification of protein structure by chemical, physical, or enzymatic means might improve the barrier properties of protein films. Enzyme-catalyzed crosslinking was useful in producing whey protein films with improved mechanical properties (Mahmoud and Savello, 1993).

Apparent water vapor permeability (WVP$_{app}$)

The ability to withstand high moisture environments is a very critical attribute of protein films. McHugh and Krochta (1994) concluded that environmental conditions such as temperature and relative humidity are major factors for film quality. WVP$_{app}$ was affected by pH of the solution. Both SD and FD wheat gluten films prepared at pH 3.3 showed higher WVP$_{app}$ than those prepared at pH 10.0. At lower pH, the unfolding of protein molecules and resulting exposure of hydrophilic groups caused an increased affinity for water molecules. Gontard et al. (1992) reported that pH and ethanol concentration had strong interac-
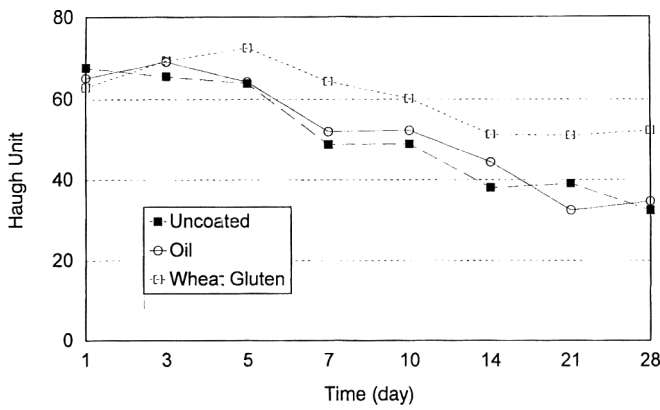
Fig. 2—Comparison of interior egg quality of uncoated and oil- and protein-coated egg shells as determined by Haugh units during 28 days of storage at room temperature.

Table 3—Effects of dipping time on water stability and strength of packaging peanuts coated with spray-dried (SD) wheat gluten solution

Dipping time (sec)	Peak force (g)	Water stability (min)
0	550.77[a]	0.25[a]
2	1169.57[b]	3.75[b]
10	1207.11[b]	4.03[b]
30	1316.99[b]	4.65[c]

[a-c] Mean values in the same column followed by different superscripts are significantly different ($P < 0.05$).

tive effects on film opacity, water solubility, and WVP_{app}, Gennadios et al. (1993) reported a significant difference in WVP_{app} of films in acidic conditions. They suggested that less intermolecular protein cross-linking occurred because of repulsive forces under acidic conditions. Salt concentration, temperature, and shearing time did not affect WVP_{app} of either SD or FD wheat gluten films.

Gluten films vs commercially available plastic wrap

Since plastic wraps are commonly used to package food products, a commercial plastic wrap was tested and compared to gluten films. The plastic wrap exhibited lower WVP_{app} than gluten films (Table 2). This could be a serious limitation for applications of protein-based, degradable films. The average WVP_{app} for wheat gluten films was 7.4 E-9 g/msPa. Gennadios et al. (1993) reported a WVP_{app} for wheat gluten films of 5.6 E-11 g/msPa at 11% relative humidity. The hydrophilic nature of protein films limits their effectiveness as water barriers. Mahmoud and Savello (1993) suggested that a glycerol × time interaction affected water vapor transferability. WVP_{app} was not affected by any other factors (Table 2). FD wheat gluten films had Young's modulus similar to that of the plastic wrap. Both FD wheat gluten film and plastic wrap were more extensible than SD wheat gluten films. Plastic wrap and pH 10.0 FD wheat gluten films were more elastic than SD wheat gluten films. Gontard et al. (1993) reported increased film extensibility from adding glycerol. However, this alteration reduced film puncture strength, elasticity, and water vapor barrier properties.

The pH 3.3 films were more plastic in nature, stretching to a greater extent before rupturing. The plastic wrap and FD wheat gluten samples exhibited lower tensile strength compared to the SD samples. Young's modulus was not different between FD wheat gluten film and the plastic wrap, suggesting that both these films were more elastic than extensible.

Wheat gluten as a coating material

SD wheat gluten was chosen for evaluation as coating material because it produced a thicker film and was more soluble

than FD wheat gluten. Shell eggs coated with SD wheat gluten solution maintained higher interior quality as measured by Haugh units (Fig. 2). After 7 days storage at room temperature, the oil-coated and uncoated shell eggs changed from Grade A to Grade B. In contrast, SD wheat gluten-coated shell eggs maintained Grade A quality throughout the study (Fig. 2).

Stability of coated packing material

Starch-based packing materials have been developed as an alternative to polystyrene packaging materials. However, decreased stability of such materials in a humid environment is a major limitation. SD wheat gluten-based film solutions were used as coating material for such starch-based packing materials. Results revealed an improvement in structural integrity and better water stability after coating (Table 3). Dipping the starch-based packaging peanuts in SD wheat gluten solution for 30 sec improved stability. "Peanuts" dipped in SD coating solution for 2, 10, and 30 sec exhibited greater strength than those not cooked as indicated by an increase peak force required. However, the extended effects were not significantly higher when the coated "peanuts" were dipped for > 2 sec.

CONCLUSIONS

WHEAT GLUTEN HAS POTENTIAL as a source of protein-based degradable films and coatings. Films prepared from SD and FD wheat gluten had differences in properties. Solubility and interactions with plasticizers are major factors influencing film properties. Films from wheat gluten compared well with plastic wrap for most properties except WVP_{app}. Gluten-based films showed promise as coating materials for perishable and semi-perishable foods such as eggs, nuts, and candies. Development of degradable films and edible coatings from wheat gluten would enhance its utility value and may provide new markets for wheat gluten.

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Zinc Chemical Form in Some Traditional Soy Foods

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———— ABSTRACT ————

The distribution and chemical form of zinc of several foods prepared from soybean were analyzed. There was a marked variation among soy foods in total zinc, water-soluble zinc, and zinc solubilized on enzymatic digestion. Chromatographic analysis indicated several zinc components were solubilized on digestion. A significant negative correlation was found for % zinc solubilized after the digestion of soy foods to the original contents of three components, i.e., protein, phosphorus and calcium. Variations among soy foods in chemical form of zinc after digestion should be taken into account on evaluating bioavailability of zinc from soy foods.

Key Words: zinc, bioavailability, soy foods, soy-meal, soy-milk.

INTRODUCTION

ZINC is an essential micronutrient for humans. A recommended dietary allowance for zinc has been established in several countries. Although widely distributed in foods, a marked variation in absorption of zinc has been observed among different foods (Sandström et al., 1989; Sandstead et al., 1982). Thus bioavailability for gastrointestinal absorption of zinc of food has attracted much attention. The bioavailability may be affected by other dietary factors such as proteins (Stuart et al., 1986), phytic acid (O'Dell, 1969), other minerals such as calcium (Forbes, 1964; Johnson et al., 1982), and dietary fiber (Reinhold et al., 1976). These findings suggest that the chemical form in which dietary zinc is presented to the absorptive cells may have a profound influence on bioavailability. Although there are previous reports on zinc following digestion of foods (Ikeda, 1990; Ikeda et al., 1990; Harzer and Kauer, 1982; Champagne and Phillippy, 1989), it remains uncertain how the chemical form prior to intestinal absorption may be related to its absorption. Especially, it is not clear what chemical forms of dietary zinc may be more or less available for absorption.

Soybean (Glycine max (L.) Merrill) is an important edible seed. Soybean contains a high level of some essential nutrients such as protein and lipid. Soybean also contains a relatively high amount of zinc, and thus may be an important dietary source. However, bioavailability of zinc of soybean for absorption is low (Sandström and Cederblad, 1980; Sandström et al., 1987). In addition, inhibitory factors responsible for reduced availability of zinc in soybean are a subject of controversy (Maga, 1982; Sandström et al., 1987; Rackis and Anderson, 1977).

A variety of soy foods are prepared by traditional processing such as soy curd, soy-milk, fermented whole soybean around the world, especially in the Eastern countries including Japan and China. It remains largely unknown whether all the zinc in such foods may be less available for absorption. Bioavailability of zinc of various traditional soy foods is of particular interest to those concerned with soybean as an important source of zinc. Chemical forms of zinc in various soy foods and their changes on digestion should be clarified.

Our objective was to analyze the distribution and chemical forms of zinc in some traditional soy foods in Japan and to identify changes in chemical forms of zinc after in vitro enzymatic digestion of the soy foods.

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MATERIALS & METHODS

Materials

Two different samples of each of various traditional soy foods were purchased from two different suppliers respectively; and 2 different samples of soybean seed (Glycine max (L.) Merrill var. Tsurunoko), purchased from 2 different suppliers. Each of the 2 different samples of soy foods and seed were randomly allocated to one of duplicate determinations. All determinations were performed in duplicate. Quadruplicate experimental data obtained by duplicate determinations of the two different samples of each of soy foods and seed were combined and subjected to statistical analysis. Eight different kinds of soy foods were studied. (The Japanese names of each soy food are expressed in italics): roasted soybean meal, Kinako; whole soybean fermented by Bacillus natto, Natto; soy-milk after squeezing soybean mash, Tonyu; an edible residue after squeezing soybean mash occurring on preparation of Tofu, Okara; soybean curd coagulated with magnesium chloride and calcium sulfate, Tofu; dried soy-milk film, rising to surface on heating of soy-milk, Yuba; freeze-dried soy curd, Kori-Tofu; and deep-fried soy curd, Abura-Age (Watanabe et al., 1974, Wang, 1984, Snyder and Kwon, 1987). The soy foods examined had been heated during commercial processing, therefore were used without further cooking. All soy samples, except for the initially-ground samples (Kinako and Okara) and a liquid sample (Tonyu), were ground into powder or paste before analysis. Three grinding procedures applicable to grinding characteristics of each soy sample were selected as follows: 4 solid soy samples (soybean seed, Yuba, Kori-Tofu and Abura-Age) were milled into powder by an electrically-driven mill; a fermented soy whole sample with a slimy appearance (Natto) was chopped up with a knife into paste-like state; and a soy curd sample (Tofu) was homogenized into paste in a mortar. Two initially-ground samples (Kinako and Okara) and a liquid sample (Tonyu) were used without treatment before analysis. An aliquot of each ground sample or liquid sample was analyzed.

Determination of mineral elements, protein and moisture

The contents of zinc, calcium and magnesium of samples were determined with a Hitachi 180-70 polarized Zeeman atomic absorption spectrophotometer. Phosphorus was assayed by the method of Fiske and Subbarow (1925). Prior to determining the total contents of these minerals, the samples were wet-ashed with sulfuric acid and 30% hydrogen peroxide prior to analysis. Protein in solid samples was estimated by the micro-Kjeldahl method (AOAC, 1984) (N $\times$ 5.71: FAO, 1947). Protein concentration was assayed by the method of Bradford (1976). The total contents of mineral elements and protein were expressed on a wet weight basis. Moisture content was determined using AOAC methods (AOAC, 1984).

Solubility of zinc, phosphorus, calcium and protein

To obtain information on the distribution of zinc, phosphorus calcium and protein in the soy foods examined, these minerals and protein present in the foods were classified with respect to water solubility. Quadruplicate samples of soy foods were extracted with 20-fold volumes (V/W) of distilled water for 2 hr at 37°C with shaking, followed by centrifugation at 10,000 rpm for 20 min. Finally, the supernatant was assayed for zinc, phosphorus, calcium and protein. Another aliquot of the supernatant was applied to a Sephadex G-50 column that was pre-equilibrated with distilled water.

In vitro proteolytic digestion

Quadruplicate samples of soy foods were subjected to in vitro proteolytic digestion according to the method of Akeson and Stahmann (1964) with a modification (Ikeda, 1984). Pepsin digestion was performed in 0.06N hydrochloric acid for 3 hr at 37°C with an enzyme-to-protein ratio

ZINC IN VARIOUS SOY FOODS . . .

Table 1—Contents of some minerals, total protein, and moisture in various soy foods[a]

Food	Zinc[b]	Phosphorus[b]	Calcium[b]	Magnesium[b]	Total protein[b]	Moisture
	mg/100g food				g/100g food	
Raw soybean meal	3.25 ± 0.31[d]	657 ± 8[e]	104 ± 4[f]	212 ± 7[c]	30.1 ± 0.2[f]	12.7 ± 0.2[h]
Kinako	3.43 ± 0.16[d]	724 ± 13[d]	127 ± 3[e]	214 ± 13[c]	36.6 ± 0.3[e]	2.0 ± 0.2[j]
Natto	1.49 ± 0.08[e]	345 ± 5[f]	70 ± 29[gh]	77 ± 3[f]	17.4 ± 0.9[g]	60.9 ± 0.5[f]
Tonyu	0.52 ± 0.04[f]	78 ± 2[h]	22 ± 1[i]	26 ± 2[g]	4.5 ± 0.1[j]	89.4 ± 0.1[c]
Okara	0.60 ± 0.05[f]	86 ± 4[gh]	50 ± 1[h]	30 ± 1[g]	5.1 ± 0.1[j]	78.0 ± 0.1[e]
Tofu	0.75 ± 0.05[f]	105 ± 1[g]	78 ± 3[g]	30 ± 1[g]	7.0 ± 0.2[i]	86.3 ± 0.6[d]
Yuba	4.08 ± 0.12[c]	707 ± 12[d]	321 ± 11[d]	195 ± 14[c]	46.2 ± 3.1[d]	7.1 ± 0.1[i]
Kori-Tofu	4.37 ± 0.09[c]	815 ± 5[c]	600 ± 16[c]	101 ± 2[e]	49.2 ± 0.7[c]	6.8 ± 0.1[i]
Abura-Age	1.83 ± 0.18[e]	363 ± 10[f]	129 ± 7[e]	162 ± 5[d]	15.8 ± 1.1[h]	49.7 ± 0.3[g]

[a] Values are means ± S.D. (n=4)
[b] On a wet weight basis.
[c-i] Mean values within a column not sharing a common superscript are significantly different at $p<0.01$.

Table 2—Some water-soluble minerals and protein content of various soy foods[ab]

Food	Zinc		Phosphorus		Calcium		Protein	
	mg/100g food (%)						g/100g food (%)	
Raw soybean meal	1.38±0.17[c]	(42.6[e])	47.1±1.1[e]	(7.2[e])	12.1±2.4[e]	(11.6[f])	3.69±0.07[c]	(12.3[d])
Kinako	0.86±0.06[d]	(25.0[f])	67.2±2.9[c]	(9.3[d])	19.5±1.3[d]	(15.4[e])	0.10±0.01[g]	(0.3[e])
Natto	0.22±0.08[ef]	(14.6[gh])	55.8±1.3[d]	(16.2[c])	4.6±0.6[fg]	(6.6[g])	0.07±0.01[g]	(0.4[e])
Tonyu	0.32±0.01[e]	(61.7[c])	7.7±0.6[gh]	(9.8[d])	11.7±0.1[e]	(53.0[c])	1.46±0.10[d]	(32.5[c])
Okara	0.31±0.02[e]	(51.9[d])	4.4±0.1[hi]	(5.1[f])	6.1±0.4[f]	(12.3[f])	0.68±0.03[e]	(13.3[d])
Tofu	0.13±0.02[ef]	(16.7[fg])	2.6±0.1[i]	(2.5[g])	22.9±1.0[c]	(29.3[d])	0.08±0.01[g]	(1.2[e])
Yuba	0.31±0.03[e]	(7.7[hi])	10.4±0.9[g]	(1.5[h])	13.3±0.9[e]	(4.1[g])	0.58±0.01[e]	(1.2[e])
Kori-Tofu	0.04±0.05[f]	(1.0[i])	14.3±0.4[f]	(1.8[gh])	2.6±0.2[g]	(0.4[h])	0.38±0.02[f]	(0.8[e])
Abura-Age	0.23±0.04[ef]	(12.6[gh])	5.3±0.4[hi]	(1.5[h])	6.8±0.8[f]	(5.3[g])	0.16±0.02[g]	(1.0[e])

[a] On a wet weight basis.
[b] Values are means ± S.D. (n=4). Values in parenthesis indicate means of per cent of each water-soluble component to the total content of soy foods.
[c-i] Values within a column that do not share a common superscript are significantly different at $p<0.01$.

of 1:100. Immediately after peptic digestion, an appropriate volume of 2M Tris-HCl buffer (pH 8.0) was added to the digestion mixtures to adjust to pH 8.0. Pancreatin solution was then added to their digestion mixtures at an enzyme-to-protein ratio of 1:20, and incubated for an additional 20 hr at 37°C (pH 8.0). The final concentration of Tris-HCl buffer in the digestion mixtures was 0.2M. Sodium azide was added to the digestion medium to a final concentration of 0.025% to prevent growth of microorganisms. Immediately after digestion, the suspensions were placed in an ice-cold vessel to diminish enzymatic action and then clarified by centrifugation (10,000 rpm, 20 min). Blank tests of the digestion were performed with the above two enzymes in the absence of the foods. The content of zinc in the soluble digesta was determined by subtraction of the food-free blank. Aliquots of the soluble digestate were applied to a Sephadex G-50 column, pre-equilibrated with 0.1M Tris-HCl buffer (pH 8.0).

Chromatography

Gel filtration chromatography was performed on a Sephadex G-50 column (1.6 × 95 cm), pre-equilibrated with 0.1M Tris-HCl buffer (pH 8.0) or distilled water. A fraction collector (Pharmacia Fine Chemicals FRAC-100) was used to collect 4.0 mL fractions that were subsequently analyzed for zinc, protein and phosphorus. The distribution of components in eluates from the chromatographic column was assayed as follows: zinc, by atomic absorption spectrophotometric procedure: protein, by A_{280} measurements; peptide, by the colorimetric method using 2,4,6-trinitrobenzenesulfonic acid (Habeeb, 1966); and phosphorus, by the method of Bartlett (1959).

Statistical analysis

Data were subjected to analysis of variance and significance of means was tested by Tukey's multiple range test ($p < 0.01$) (Steel and Torrie, 1980).

RESULTS & DISCUSSION

Contents of mineral elements and protein in various soy foods

The contents of zinc, phosphorus, calcium, magnesium, total protein and moisture on a wet weight basis were compared in various soy foods (Table 1). There was a variation in total zinc content among soy foods examined: it ranged from 0.52 mg to 4.37 mg/100g fresh weight. Kori-Tofu contained the highest

level of zinc and Tonyu, the lowest. There was also variation in other minerals.

Distribution and chemical forms of zinc in various soy foods

Water-soluble zinc, phosphorus, calcium and protein were compared on various soy foods (Table 2). A variation in water-soluble zinc was noted, with the % water-soluble zinc in a range from 1.0% to 61.7%. Raw soybean meal, Tonyu and Okara had a high proportion of water-soluble zinc to total zinc; whereas Natto, Tofu, Yuba and Abura-Age, a low proportion. Less water-soluble zinc was found in Kori-Tofu, freeze-dried Tofu, as compared with Tofu. This was interesting because Kori-Tofu is made by freeze-drying Tofu. Saio (1973) suggested that the sponge-like texture of Kori-Tofu may be prepared from Tofu, through the formation of disulfide bonds of soy protein during drying. In view of zinc nutrition, it was noticed that the majority of zinc in Kori-Tofu became insoluble, perhaps through binding to insoluble components such as protein, during drying.

Chromatographic elution profiles of water extracts of various soy foods were compared on Sephadex G-50 (Fig. 1). Most water-soluble zinc in raw soybean meal eluted in a fraction with a molecular weight of 1 to 2 kDa (A in Fig. 1). A similar result was observed with Kinako (B in Fig. 1), Tofu (D in Fig. 1) and Yuba (E in Fig. 1). Unlike raw soybean meal, the majority of water-soluble zinc in Natto (C in Fig. 1) and Kori-Tofu (F in Fig. 1) was eluted with several different components. The water-soluble zinc of Kori-Tofu emerged as five distinguishable components (F in Fig. 1), whereas most of water-soluble zinc of Natto distributed in several non-distinguishable components with molecular weights of around 1 to 8 kDa (C in Fig. 1). The results (Table 2 and Fig. 1) suggest that processing of soybean to its various products affects the distribution and chemical forms of zinc in the products.

Zinc released on *in vitro* digestion of soy foods

Percent zinc released after the in vitro peptic and pancreatic digestion of various soy foods was compared (Table 3). A significant ($p < 0.01$) variation in percent zinc released on diges-
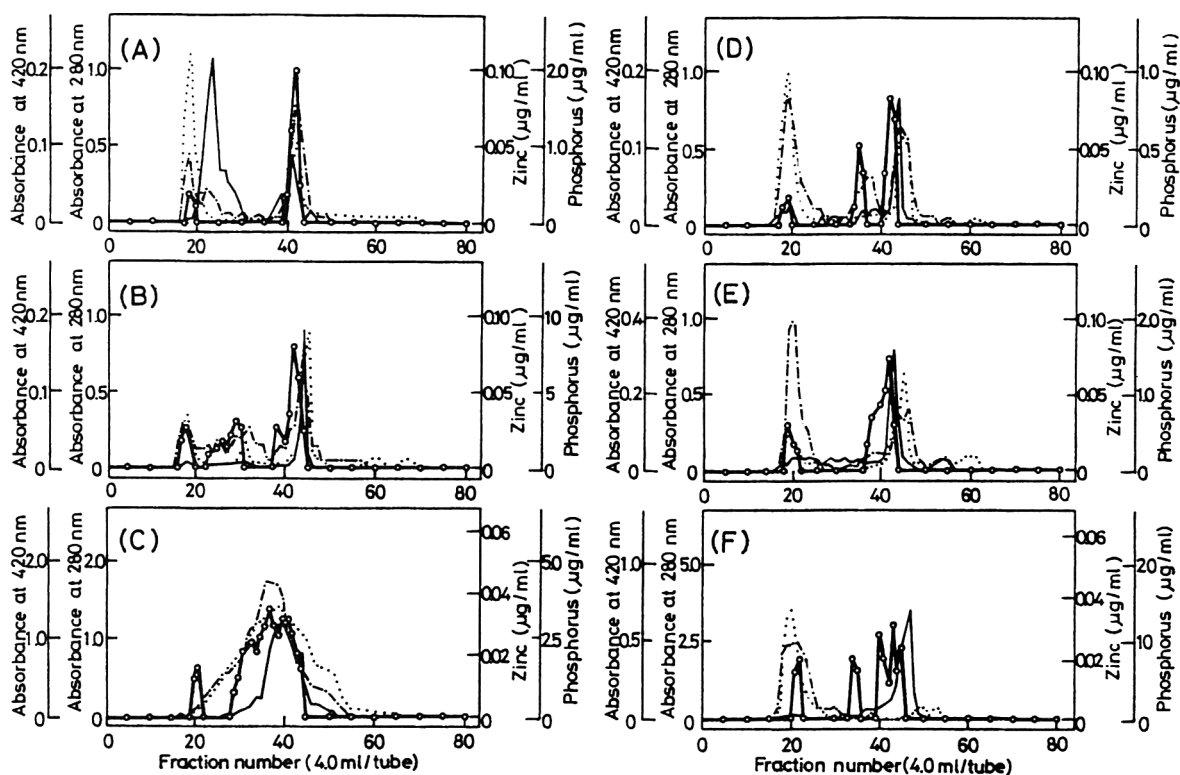
Fig. 1—Gel filtration chromatographic elution profiles of water extracts of various soy foods on Sephadex G-50. (A) Raw soybean meal; (B) *Kinako*; (C) *Natto*; (D) *Tofu*; (E) *Yuba*; (F) *Kori-Tofu.* ——o——, Zinc;———, phosphorus;·······, absorbance at 280 nm; and —·—·—·—, absorbance at 420 nm after incubation with TNBS.

tion was found among soy foods, with the percent soluble zinc ranging from 2.7% to 97.0%. A high percent of zinc was released on digestion of *Tonyu, Okara* and *Tofu*, whereas a low percent of zinc was released on digestion of *Yuba* and *Kori-Tofu*. Generally, bioavailability of minerals for absorption is closely associated with its solubility in the intestinal tract. These findings indicate that the enzymatic digestion of soy foods, except for raw soybean meal and *Kori-Tofu*, increases the solubility of zinc. On the other hand, although the zinc in some soy foods, i.e. *Yuba, Abura-Age, Kinako*, have low solubility after digestion, these foods may contribute more bioavailable zinc on an absolute basis to the diet than foods with highly soluble zinc, i.e. *Tonyu, Okara*.

Zinc released on digestion as related to other components

Relationships of percent zinc released on the in vitro digestion of soy foods to total protein (A in Fig. 2), total phosphorus (B in Fig. 2), total calcium (C in Fig. 2) and total magnesium (D in Fig. 2) were compared on a wet weight basis. Interestingly, there was a significant ($P < 0.01$) negative correlation between % zinc released on digestion of soy foods (Table 2) and their original total protein contents, correlation coefficient -0.88 (A in Fig. 2). There was also a significant ($P < 0.01$) negative correlation between % soluble zinc released on digestion of soy foods (Table 2) and their original total phosphorus, correlation coefficient -0.87 (B in Fig. 2). A weakly but significant ($P < 0.05$) correlation between percent soluble zinc released on digestion and the original content of total calcium was also found, correlation coefficient -0.78 (C in Fig. 2). Substantially no correlation was found between % soluble zinc released on digestion and the original content of total magnesium (D in Fig. 2).

Bioavailability of zinc from soybeans is low (Sandström and Cederblad, 1980; Sandström et al., 1987). Some dietary factors such as protein, phytic acid, some minerals and dietary fiber, may adversely affect zinc bioavailability (Sandström et al., 1987; Stuart et al., 1986; O'Dell, 1969; Zhou et al., 1992;

Table 3—Zinc released on the in vitro digestion of various soy foods

Food	Zinc released on digestion[ab] mg zinc/100g food (%)
Raw soybean meal	1.38 ± 0.05^c (42.4[f])
Kinako	1.41 ± 0.03^c (41.2[f])
Natto	0.53 ± 0.08^{ef} (35.4[f])
Tonyu	0.51 ± 0.03^{ef} (97.0[c])
Okara	0.42 ± 0.02^f (70.1[e])
Tofu	0.61 ± 0.03^{de} (80.7[d])
Yuba	0.62 ± 0.08^{de} (15.19)
Kori-Tofu	0.12 ± 0.02^g (2.7[h])
Abura-Age	0.70 ± 0.06^d (38.5[f])

[a] On a wet weight basis.
[b] Values are means $\pm$ S.D. (n=4). Values in parenthesis indicate means of percent of each zinc released on the digestion of soy foods to their total zinc contents.
[c-h] Values within a column that do not share a common superscript are significantly different at $p<0.01$.

Forbes, 1964; Johnson et al., 1982; Reinhold et al., 1976). Phytic acid may inhibit absorption of dietary zinc in the intestinal tract through its ability to bind and precipitate zinc (O'Dell, 1969). However, no conclusive evidence for clarification of the mechanism responsible for reduced availability of zinc in the gastrointestinal tract has been reported. In addition, phytic acid accounts for the majority of the total phosphorus in soybean (Erdman, 1979; Reddy et al., 1982). Considerable attention has been substantially paid to improve zinc availability by reduction of phytic acid from soybean (Zhou et al., 1992; Moeljopawiro et al., 1988). A negative correlation of % zinc released on digestion to the total phosphorus (B in Fig. 2) was of interest. Especially, *Kori-Tofu* had the lowest level of zinc released on digestion among the soy foods examined (Table 3). The reduced % zinc released in *Kori-Tofu* may be attributable to the presence of the highest levels of total phosphorus (Table 1). Evidence suggests that the protein of soybean may reduce assimilation of endogenous zinc (Sandström and Cederblad, 1980; Sandström et al., 1987). In addition, zinc of soybean may become less available by formation of a phytate-protein-zinc complex (Rackis and Anderson, 1977). The Standard Tables of Food
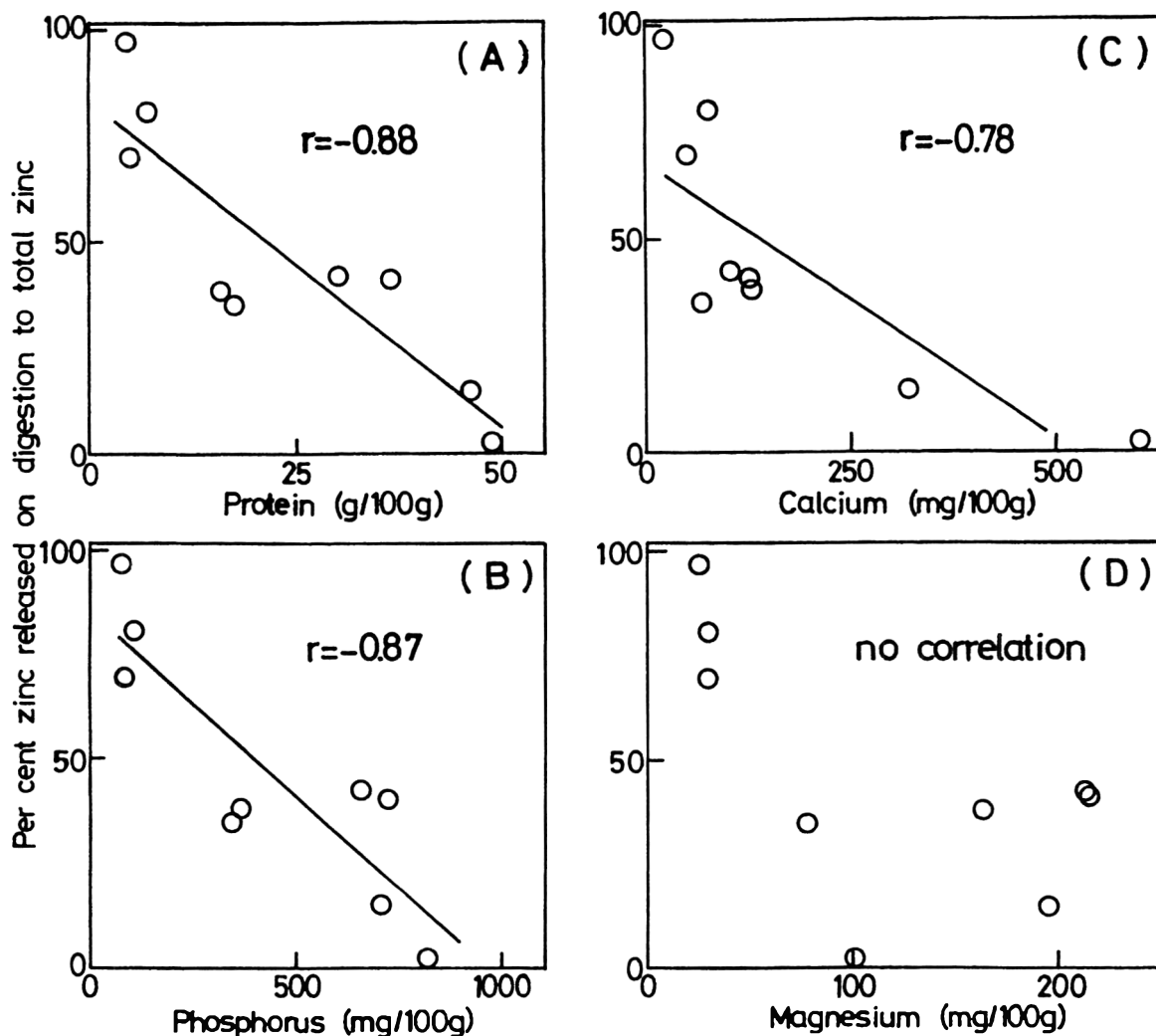
Fig. 2—Relationships between percent zinc released on the *in vitro* digestion of soy foods to each total zinc, and the original contents of their some components. (A) Total protein; (B) total phosphorus; (C) total calcium; and (D) total magnesium. The contents of all the components were expressed on a wet weight basis.

Composition in Japan (Resources Council, Science and Technology Agency, Japan, 1984) have shown a variation in protein digestibility by humans among traditional soy foods, e.g., *Yuba* exhibited higher protein digestibility; and *Kinako*, lower. Therefore, there was substantially no correlation of observed, percent zinc released on the digestion of soy foods (Table 3) to the previously-reported protein digestibility on humans (Resources Council, Science and Technology Agency, Japan, 1984). This suggests that solubilization of zinc on digestion of soy foods may be not directly associated with protein digestibility. However, it is known that calcium supplementation of corn-soy hog rations can cause zinc deficiency in hogs (Tucker and Salmon, 1955). Many other studies with animals have also indicated that calcium reduced the availability of zinc of soybean (Forbes, 1964; Heth and Hoekstra, 1965). The observed correlation of percent zinc released on digestion of soy foods to their total calcium contents (C in Fig. 2), agreed with previous in vivo reports.

Our study (Fig. 2) indicated that % zinc released on digestion of soy foods to each total zinc exhibited a significantly negative correlation to the original contents of total protein, phosphorus and calcium. These findings suggest that protein, phosphorus and calcium may be important factors affecting solubilization of zinc in the gastrointestinal tract. The negative correlations reported here (A to C in Fig. 2) agreed with in vivo studies, which show inhibitory effects on zinc absorption of phytic acid (O'Dell, 1969), protein (Sandström et al., 1987; Stuart et al.,

1986) and calcium (Tucker and Salmon, 1955; Heth and Hoekstra, 1965; Likuski and Forbes, 1965).

Distribution of zinc released on the digestion of various soy foods

Chromatographic elution profiles of the soluble digests from various soy foods on *in vitro* enzymatic digestion were compared on Sephadex G-50 (Fig. 3). Zinc in the soluble digests of all soy foods (A to F and H in Fig. 3). except for *Kori-Tofu* (G in Fig. 2), eluted with one major protein-like peak having a molecular weight of 1 to 2 kDa. A variation in compositions, including peptide and phosphorus, of the observed major zinc peaks among various soy foods (A to F and H in Fig. 3) was observed. Zinc in the soluble digests of *Kori-Tofu* consisted of 4 different peak fractions with molecular weights of 1 to 2 kDa, 4 kDa, 5 kDa and 22 kDa respectively (G in Fig. 2).

Current evidence suggests that some amino acids such as histidine and cysteine may improve absorption by forming soluble complexes with zinc and thus preventing formation of insoluble hydroxides (Greger, 1989). Some dietary constituents such as phytic acid and calcium may exhibit inhibitory effects on zinc absorption through coprecipitating with zinc (Sandstead, 1982).

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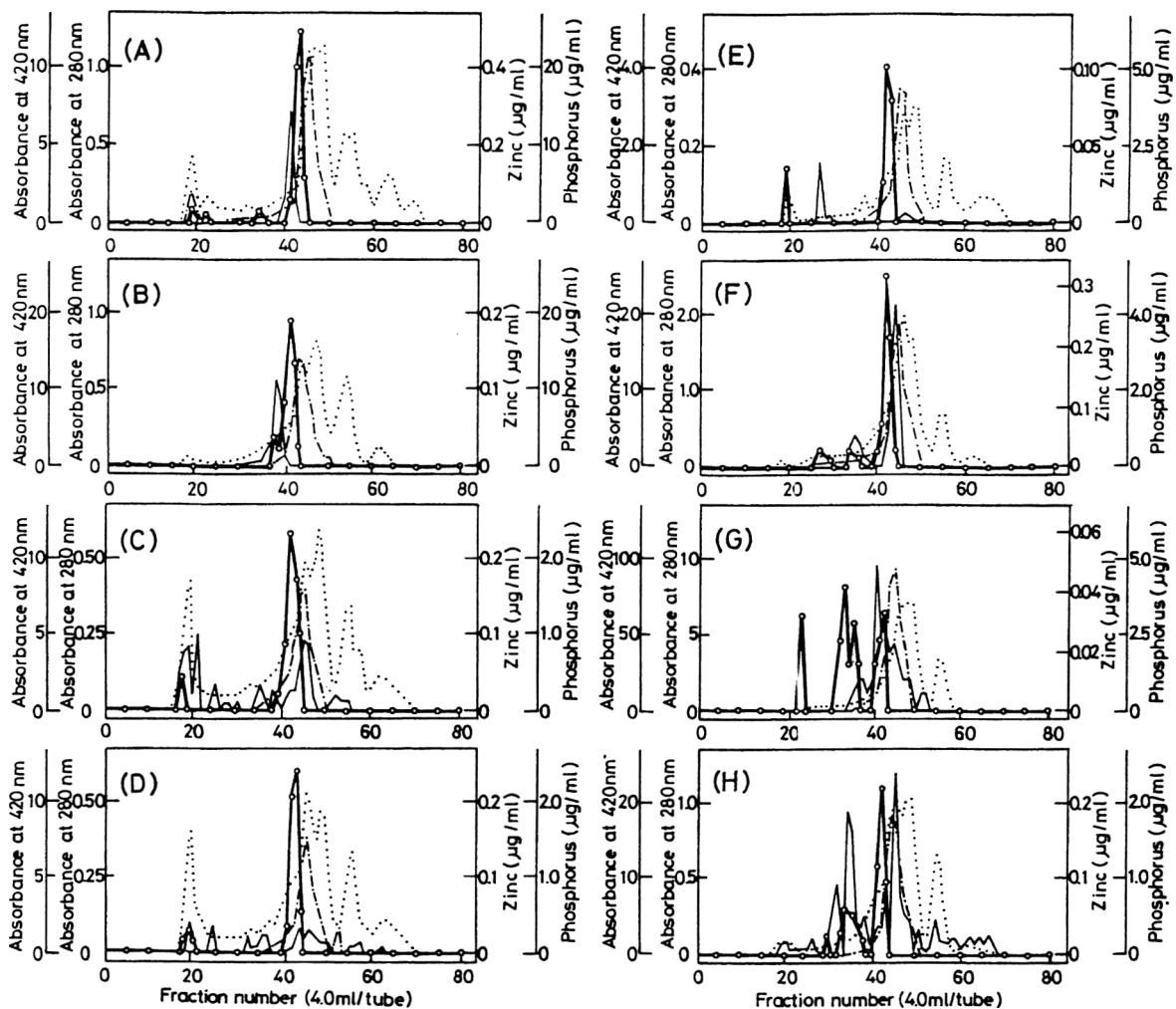
Fig. 3—Gel filtration chromatographic elution profiles of the soluble digesta from various soy foods on *in vitro* enzymatic digestion on Sephadex G-50. (A) Raw soybean meal; (B) *Natto;* (C) *Tonyu;* (D) *Okara;* (E) *Tofu;* (F) *Yuba;* (G) *Kori-Tofu;* (H) *Abura-Age.* ———O———, Zinc;———, phosphorus; ·······, absorbance at 280 nm; and —·——·—, absorbance at 420 nm after incubation with TNBS.

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Ms received 11/25/94, revised 3/31/95; accepted 4/15/95.

ERRATUM NOTICE

•*J. Food Science* (Vol. 60, No. 3, 1995), pages 541-546—Ascorbic acid and 5-methyltetrahydrofolate losses in vegetables with cook/chill or cook/hot-hold foodservice systems by Peter G. Williams, Helena Ross, and Jennie C. Brand Miler. Dr. P.G. Williams, senior author, advises that when two tables were combined at the request of the reviewers, the "Warm-holding" data (last two columns of Table 11, page 545) were not reordered to correspond correctly with the vegetables as listed. The corrected version of Table 11 is printed herewith, with apology from the authors for any inconvenience to JFS readers.

Table 11—Vitamin losses in cooked vegetables during chilled storage (%/day) and warm-holding (%/hr)

Vegetable	Chilled storage (3°C)		Warm-holding (71°C)	
	Vitamin C	5MeTHF	Vitamin C	5MeTHF
Carrots	14.9	6.7	33.1	10.3
Pumpkin	10.8	9.9	23.8	13.8
Mashed potato	8.4	6.7	24.1	15.2
Steamed potato	8.2	6.4	15.5	9.5
Silverbeet	7.3	1.5	28.3	27.7
Broccoli	3.3	0.3	29.6	13.6
Peas	2.2	1.9	30.8	8.0
Mean	7.9	4.8	26.4	14.0

Guidelines for the Preparation and Review of
Seafood Technology-Related Papers
by the IFT Seafood Technology Division

□The Seafood Technology Division of the Institute of Food Technologists has prepared the following guidelines for use by authors in preparing seafood technology-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. These guidelines—prepared by Joe M. Regenstein and approved by the Seafood Technology Division executive committee—are intended to supplement the "Style Guide for Research Papers" published in *Journal of Food Science* 59: 1367-1370 (1995).

Papers reporting seafood technology-related data should include the following information:

I. Species studied

A. Common name (local name)
B. Market name (from the Food and Drug Administration List)
C. Genus and species (if recently changed, then also previous name, e.g. *Salmo gairdneri* to *Onchorhynchus mykiss* for rainbow trout and steelhead)

II. Harvesting information

A. Location
B. Time of year
C. If available, type of equipment used to catch
D. Whether bled at sea
E. Whether gutted at sea
F. Physical condition of the fish (e.g. feeding or spawning, fat or starved)
G. Size of fish (weight and length)
H. Whether any special way of "killing/stunning" was used

III. Approximate age of fish

Year class
Time since caught
If commercial, source
How transported to the lab

IV. How stored (e.g., ice in 0–2°C cooler, ice in 2–4°C cooler)

A. Ice
B. Ambient environment

V. Processing history

At what stage was it gutted? Was the belly fully cleaned, e.g., the swim bladder removed? Was the kidney tissue actively removed? Was the black lining of the belly flaps removed? Provide other processing history in sufficient detail to understand what was done to the fish.

VI. Additional information for aquacultured fish

A. Formulation of diet(s)
B. Whether fed or fasted before harvest
C. Water temperature
D. Active slaughter? If so, what?
E. Dissolved oxygen in the water
F. Recirculating system vs single pass. More information about the source and quality of the water
G. Any drugs used on the fish
H. Other husbandry issues that might affect the quality of the fish
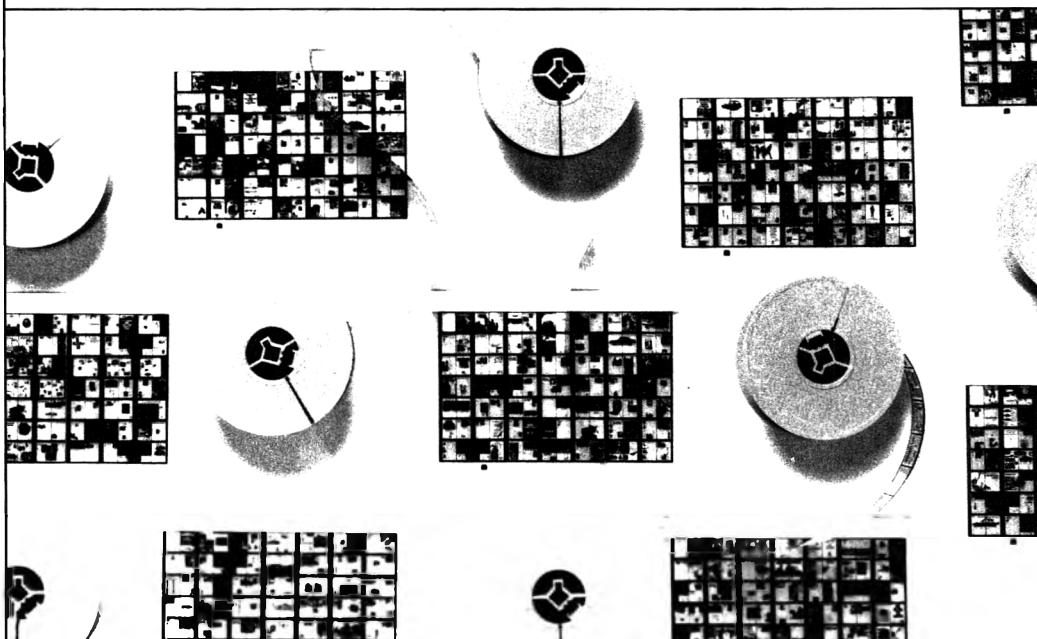
VII. Portion of the fish sampled

VIII. Additional information that may be helpful

A. Were matched fillets used?
B. If available, the real fluctuation in the storage unit, e.g., in the freezer
C. Details about salt, beyond just the words "sea salt:" Some idea of the standard the salt met. For salt fish experiments, some microbiological information about the quality of the salt is necessary
D. More details of the microbiology: Consideration of adding salt to the media and why the particular temperature was used for standard plate counts
E. Whether fillets are boneless and/or skinless. Also what parts were trimmed. Identify steaks by their thickness and whether samples are from before the anus (i.e., with part of the belly flap) or after the anus
F. Packaging: Material used, thickness, vacuum, etc.

IX. Appropriate vs inappropriate content for Journal of Food Science

A. By-product utilization studies are appropriate
B. Use of products in animal diets is probably inappropriate

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